ATTACHMENT - REMARKS

Claims 1-11 are pending in the present application. By this Amendment, Applicants have amended claim 5. Applicants respectfully submit that the present application is in condition for allowance based on the discussion which follows.

In the outstanding Office Action, claims 3-5 were rejected under 35 U.S.C. § 112, first paragraph, alleging that the specification, while being enabling for treating malignant melanomas with PRIMA-1, does not necessarily provide enablement for inhibiting undesired angiogenesis.

Contrary to the enablement rejection to claims 3-5 under 35 U.S.C. § 112, first paragraph, Applicants respectfully submit that one of ordinary skill in the art would be able to practice the invention, as claimed, based on the disclosure in the specification and what one of ordinary skill in the art would know. Filed contemporaneously with this Amendment is a Declaration from co-inventor Staffan Strömblad, Ph.D. (hereinafter "Strömblad Dec."), which provides evidence of what one of ordinary skill in the art would understand with regard to the claimed method. Moreover, the Strömblad Dec. provides evidence establishing that one of ordinary skill in the art, in view of the present specification, would be enabled to practice the claimed method to inhibit undesired angiogenesis (see Strömblad Dec.).

The present invention describes experiments which convincingly show:

(1) wild type p53 may be present in the cell in an inactive conformation despite being non-mutated,

(2) the inactivation of wild type p53 is mediated by the integrin $\alpha_v\beta_3$, and

(3) the inactive conformation of wild type p53, in the presence of integrin $\alpha_v\beta_3$, may be restored by the novel compounds of the present invention (see Strömblad Dec., page 1, fourth paragraph-page 2, first paragraph).

One of ordinary skill in the art, based on the present specification, and in view of what is previously known in the art, would be able to practice the invention as claimed. In two papers, viz. Strömblad et al., J. Clin. Invest. 98:426-433 (1996) (hereinafter "Strömblad (1996)," Appendix A to these remarks); and Strömblad et al., J. Biol. Chem. 277: 13371-13374 (2002) (hereinafter "Strömblad (2002)," Appendix B to these remarks) referred to at page 7 in the present specification, the involvement of p53 and integrin $\alpha_v\beta_3$, respectively, in angiogenesis is discussed (Strömblad Dec., page 2, first full paragraph).

Thus, these papers together teach that in neovascularisation of wild type animals, α_v-integrins, such as $\alpha_v\beta_3$, are activated in the proliferative endothelial cells and this activation leads to a suppression of the activity of p53 (Strömblad Dec., page 2, first full paragraph). Furthermore, it is shown that when the integrins are blocked, by use of integrin antagonists, endothelial p53 activity is restored and the vascular cells undergo apoptosis (Strömblad Dec., page 2, first full paragraph). Further, it is shown in p53 null mice that angiogenesis is refractory to inhibition of α_v-integrins, thus functionally linking *in vivo* the $\alpha_v\beta_3$-mediated inactivation of p53 in vascular cells to their capacity to undergo angiogenesis (Strömblad Dec., page 2, first full paragraph).

These results provide evidence that the cell survival of both malignant melanoma cells and endothelial cells undergoing angiogenesis is under the influence of integrin $\alpha_v\beta_3$, suppressing the activity of p53 (Strömblad Dec., page 2, first full paragraph).

As pointed out herein above, in the present application, the present inventors have shown that the apoptosis-inducing activity of wild type p53, lost in the presence of $\alpha_v\beta_3$ (i.e., inactive wild type p53), may be restored by treatment with the present inventive compounds (Strömblad Dec., page 3, second paragraph). In view of the teachings of the two prior art documents referred to herein above, it is contemplated that this same principle may also be applied in endothelial cells undergoing angiogenesis under the influence of $\alpha_v\beta_3$ and the inventive compounds, therefore, also may be applied in a method of treating undesired angiogenesis (Strömblad Dec., page 3, second paragraph-page 7, first full paragraph).

The majority of mortality associated with cancer is due to the metastasis of the original tumor cells, and angiogenesis is vital to the metastazation process. The reason is that the tumor formed by the metastazing cells needs a supply of nutrients and oxygen in order to grow, which requires the formation of new blood vessels through angiogenesis induced by the tumor cells; cf., e.g., Strömblad et al. (2002; *vide supra*), page 13373, right column, bottom paragraph. Therefore, by the method of inhibiting undesired angiogenesis according to the present invention, a novel, efficient way of combating cancer metastazation is provided.

Undesired angiogenesis, however, also is associated with other diseases. For example, it is a major cause of blindness in various ocular diseases, such as diabetic retinopathy and the wet form of age-related macular degeneration.

In view of the above, it is respectfully submitted that the presently claimed invention provides an important new way of treating persons suffering from disorders associated with undesired angiogenesis or malignant melanoma. This contribution to

medicine will be of great value to the increasing number of patients suffering from malignant melanoma, as well as patients suffering from other life threatening or seriously disabling diseases andm accordingly, a novel and non-obvious advancement over prior methods fully enabled for one of ordinary skill in the art to practice.

Based on the foregoing, Applicants respectfully submit that claims 3-5 are in full compliance with the enablement requirements under 35 U.S.C. § 112, first paragraph.

Claims 3-5 were rejected under 35 U.S.C. § 103 as being obvious from Bykov et al. (hereinafter "Bykov") in view of Hartmann et al. (hereinafter Hartmann").

Contrary to the obvious-type rejection, Applicants respectfully submit that in view of the prior discussion describing the present method, and in view of the following discussion, Applicants respectfully submit that claims 3-5 are not obvious from Bykov in view of Hartmann.

The present invention is directed to a novel method for treating malignant melanomas based on treating individuals which have malignant melanoma cells producing inactive yet wild type p53, which results from an inactive conformation of wild type p53. By this Amendment, Applicants have amended claim 5 to even more clearly recite that the individuals have malignant melanoma cells which produce inactive yet still wild type p53 which is inactive as a result of an inactive conformation.

In sharp contrast to the present method, both Bykov and Hartmann are directed to **mutant** p53, that is p53 having lost its normal function due to a mutation in the gene sequence. Neither disclose wild type p53 which is an inactive form. One of ordinary skill in the art would readily appreciate that mutant p53 and inactive wild type p53 are two completely different compounds. One would not refer to wild type p53 which has

lost its inactivity as "mutant p53," as the term mutant is specifically used in this art to refer to a mutation at the gene sequence level (see Bykov et al., "Restoration of the tumor suppressor function to mutant p53 by a low-molecular-weight compound" (2002) (hereinafter "Bykov (2002)", Appendix C to these remarks). One would not refer to an inactivation due to conformational change of the gene product, e.g., in this case, the gene product p53, as mutant p53.

Novelty and non-obviousness of the present invention lies, in part, based on a surprising finding that, although a cell may produce wild type p53, the wild type p53 may lose its normal functionality.

Furthermore, the mechanism in which wild type p53 has lost its normal function and the mechanism by which mutant p53 has lost its function are by a completely different mechanism. Accordingly, one of ordinary skill in the art would not expect that a treatment which corrects or addresses mutant p53 to establish normal functioning p53 would have a similar effect in restoring function to inactive wild type p53 since the mechanism by which each respective p53 is inactive is completely different from one another. Thus, the appearance of an inactive conformation in wild type p53 is in no way linked to a mutation in the gene sequence of p53 (i.e., mutant p53). This is indeed a surprising finding. Moreover, the newly discovered mechanism of the cause of inactive wild type p53, i.e. not by a gene mutation, is quite significant and directly leads to a surprising trait which underlies the present invention. Therefore, one would not have expected that a treatment targeting mutant p53 would in any way have any effect on inactive wild type p53.

Moreover, in malignant melanoma, mutant p53 plays a minor role as has been previously discussed by the Applicants in prior responses and has been recognized by the Examiner. However, in developing the present invention, the present inventors found that a loss of activity of wild type p53 is generally involved in the occurrence and growth of malignant melanoma cells. Prior to this present invention, a link between a loss of activity of wild type p53 and the occurrence and growth of malignant melanoma cells was not known. It was through extensive experimentation that the present inventors were able to show that the loss of activity of wild type p53 is due to a loss in conformation mediated by the action of integrin $\alpha_v\beta_3$. These experiments have permitted the present inventors to make the conclusion, as described in detail in the present specification at pages 10-14 under the heading "Experimental" and in Figures 1A-3E.

First, by experiments described at pages 12-13, and illustrated in Figures 2A-D, the present inventors show that the integrin $\alpha_v\beta_3$ induces an unfolded conformation of wild type p53 and suppresses wild type p53 activity. Thus, experiments are performed wherein the specific DNA binding activity of wild type p53 is compared between melanoma cells expressing integrin $\alpha_v\beta_3$ or lacking expression of integrin $\alpha_v\beta_3$. It is shown that integrin $\alpha_v\beta_3$ inhibits wild type p53 activity without influencing wild type p53 protein levels.

Next, by experiments described in the present specification at pages 13-14 and Figures 3A-D, the present inventors show that integrin $\alpha_v\beta_3$-mediated suppression of wild type p53 activity leads to melanoma cell survival and melanoma tumor growth. In particular, experiments were performed *in vivo*, by injection of human melanoma cells in

mice (cf. last two lines of page 13 and page 14). As a result, Figure 3C shows the tumor growth in mice having received injection of different clones of melanoma cells (1×10^6). In Figure 3C:

(1) M21 is a melanoma cell expressing wild type p53 and lacking expression of $\alpha_v\beta_3$;

(2) M21L is a melanoma cell expressing wild type p53 but not expressing $\alpha_v\beta_3$; and

(3) Lp53His175-0 and Lp53His175-8 are melanoma cells having expression of dominant negative p53 specifically blocking the action of endogenous p53.

From Figure 3C, it appears that the growth of melanoma cells expressing wild type p53 and of $\alpha_v\beta_3$ is essentially identical to that of melanoma cells having blocked p53, whereas the growth of melanoma cells having wild type p53 and lacking expression of $\alpha_v\beta_3$ is substantially lower. Thus, inactivation of wild type p53 mediated by $\alpha_v\beta_3$ leads to growth of melanoma cells in mice.

The present inventors then went on to show that the inactivation of wild type p53 may be reversed by use of the compounds of the invention, and that this may be used to induce apoptosis of melanoma cells, in a series of experiments described in the present specification at pages 15-17 and in Figures 4A-4H.

Thus, melanoma cells having wild type p53 and expressing or lacking expression of the integrin $\alpha_v\beta_3$ were incubated with or without the inventive compound PRIMA-1 for 7 days and analysed by means of antibodies specific for wild type p53 in inactive

conformation (PAb240) and for wild type p53 in active conformation (PAb1620), respectively.

In Figure 4A, the percent reactivity of the melanoma cells with the respective antibodies are illustrated.

At the left hand side of Figure 4A, results are shown for cells expressing both wild type p53 and $\alpha_v\beta_3$, in the absence (Control) or presence of PRIMA-1, respectively. In the absence of PRIMA-1, the reactivity to antibodies recognizing wild type p53 in active conformation is close to zero, while there is a substantial reactivity to antibodies recognizing wild type p53 in inactive conformation. In the presence of PRIMA-1, on the other hand, the reactivity to antibodies recognizing wild type p53 in inactive conformation is close to zero, while the reactivity to antibodies recognizing wild type p53 in active conformation is substantially increased. This provides evidence that in melanoma cells expressing both wild type p53 and $\alpha_v\beta_3$, PRIMA-1 is capable of inducing the active conformation of wild type p53 despite the presence of $\alpha_v\beta_3$.

At the right hand side of Figure 4A, results are shown for cells expressing wild type p53 but lacking expression of $\alpha_v\beta_3$ in the absence (Control) or presence of PRIMA-1, respectively. In these cells, wild type p53 are recognized by the antibody specific for the active conformation in both the presence and absence of PRIMA-1. This indicates that in cells expressing wild type p53 but lacking expression of $\alpha_v\beta_3$, wild type p53 is in its active conformation irrespectively of the presence or absence of PRIMA-1.

Finally, the present inventors also have confirmed that the restoration of an active wild type p53 conformation by PRIMA-1 leads to induction of apoptosis of melanoma cells *in vivo* in mice, so as to block melanoma tumor growth. This experiment

is described at page 16, penultimate paragraph to page 17, first paragraph and illustrated in Figures 4G and 4H.

In Figure 4G (upper diagram), the tumor volume in mice after injection of 1.5×10^6 human melanoma cells expressing both wild type p53 and $\alpha_v\beta_3$ is shown. In mice receiving only PBS, the tumor volume was about 300 mm3 at the end of the period, while in mice receiving PRIMA-1, the tumor volume was about 100 mm3.

Thus, the results show that PRIMA-1 effectively blocked tumor growth in mice having received human melanoma cells expressing both wild type p53 and $\alpha_v\beta_3$, and that in the absence of PRIMA-1, wild type p53 did not seem capable of blocking growth of tumors.

To summarize: the present inventors have found a new mechanism explaining why, in malignant melanoma cells, p53 is non-functional even when being non-mutated, i.e. wild type. This mechanism involves the presence of the integrin $\alpha_v\beta_3$. Having found this mechanism, the present inventors have been able to show that that functionality of the wild type p53 may be restored even in the continued presence of the integrin $\alpha_v\beta_3$. Finally, the inventors have been able to show that by restoring the functionality of the wild type p53, malignant melanoma may be successfully treated.

It should be noted that in the switch to vertical growth phase, the melanoma cells acquire expression of the integrin $\alpha_v\beta_3$ (Albelda et al. Cancer Res 1990, attached as Appendix D to these remarks). This implies that $\alpha_v\beta_3$-mediated inactivation of wild type p53 is a general mechanism in malignant melanoma.

It also should be pointed out that in the experiments, the nucleotide sequence of the wild type p53 had been determined by sequencing and it was confirmed that it was not mutant.

Determining the sequence of a gene, such as the p53 gene, is a matter of routine experiment. Before the present invention, the person of skill in the art, having determined the p53 genotype of the melanoma cells of a person suffering from a malignant melanoma and found that it was of the wild type, would have had no motivation to treat the person with a compound known to act on *mutant* p53.

Based on the foregoing, Applicants respectfully submit that claims 3-5 are not obvious from Bykov in view of Hartmann.

In view of the foregoing, Applicants respectfully submit that the present application is in condition for allowance.

Respectfully submitted,

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APPENDIX A

Suppression of p53 Activity and p21$^{WAF1/CIP1}$ Expression by Vascular Cell Integrin αvβ3 during Angiogenesis

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Abstract

Induction of p53 activity in cells undergoing DNA synthesis represents a molecular conflict that can lead to apoptosis. During angiogenesis, proliferative endothelial cells become apoptotic in response to antagonists of integrin αvβ3 and this leads to the regression of angiogenic blood vessels, thereby blocking the growth of various human tumors. Evidence is presented that administration of αvβ3 antagonists during angiogenesis in vivo selectively caused activation of endothelial cell p53 and increased expression of the p53-inducible cell cycle inhibitor p21$^{WAF1/CIP1}$. In vitro studies revealed that the ligation state of human endothelial cell αvβ3 directly influenced p53 activity and the bax cell death pathway. Specifically, agonists of endothelial cell αvβ3, but not other integrins, suppressed p53 activity, blocked p21$^{WAF1/CIP1}$ expression, and increased the bcl-2/bax ratio, thereby promoting cell survival. Thus, ligation of vascular cell integrin αvβ3 promotes a critical and specific adhesion-dependent cell survival signal during angiogenesis leading to inhibition of p53 activity, decreased expression of p21$^{WAF1/CIP1}$, and suppression of the bax cell death pathway. (*J. Clin. Invest.* 1996. 98:426–433). Key words: endothelial cells • gene expression • apoptosis • cell cycle • bcl-2

Introduction

Angiogenesis is a critical process during development, wound healing, and various diseases including cancer, adult blindness, and inflammatory disorders (1). We recently showed that integrin αvβ3 becomes expressed on angiogenic vascular cells where it plays a critical role in angiogenesis induced by basic Fibroblast growth Factor (bFGF)[1], TNF-α, or fragments of human tumors in the chick embryo (2, 3). In fact, antagonists of αvβ3 administered intravenously to chick embryos caused regression of angiogenic blood vessels on the chorioallantoic membrane (CAM) due to unscheduled apoptosis (2). Importantly, this resulted in the regression of human tumors on the chick CAM or in human skin transplants on SCID mice (2, 4).

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1. *Abbreviations used in this paper:* bFGF, basic fibroblast growth factor; CAM, chorioallantoic membrane; EMSA, electrophoretic mobility shift assay; HUVEC, human umbilical vein endothelial cells; TNA, total nucleic acids.

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Cell survival and proliferation depend on appropriate signals mediated by growth factors and/or adhesion proteins within the extracellular matrix (5, 6). However, incompatible signaling events can result in apoptosis (7–12). We hypothesized that during angiogenesis, integrin αvβ3 promotes an adhesion-dependent survival signal necessary for normal cell cycle progression, since antagonists of αvβ3 administered in vivo preferentially caused apoptosis of proliferating vascular cells (2). Therefore, we examined whether the observed apoptosis might be associated with the induction of conflicting signals during endothelial cell cycle progression. Recent reports have demonstrated that cells expressing active p53 while undergoing DNA synthesis in vitro become apoptotic, presumably due to conflicting signals (7–11). Induction of apoptosis by p53 has been associated with its ability to promote or repress transcription of genes that either influence cell cycle progression or directly regulate cell survival and apoptosis (13–16). Therefore, experiments were designed to evaluate whether ligation of endothelial cell αvβ3 influenced expression and/or activity of p53 and of putative mediators of p53-induced apoptosis during angiogenesis.

Methods

Cell lines and tissue culture. Human umbilical vein endothelial cells (HUVECs) from pooled donors (Clonetics Corp., San Diego, CA) were grown in M199 containing 20% FBS, 100 μg/ml gentamicin, 4 mM L-glutamine, 0.9 mg/ml heparin and 30 μg/ml endothelial cell growth supplement (Upstate Biotech. Inc., Lake Placid, NY).

Cell adhesion to antiintegrin antibodies as agonists of integrin function. For evaluation of effects by individual integrins, plastic dishes were first coated with 25 μg/ml goat anti-mouse polyclonal antibodies (Jackson ImmunoResearch Labs., Inc., West Grove, PA) at 37°C for 2 h, followed by blocking with 1% heat-denatured BSA at 37°C for 1 h. Then, mouse monoclonal antibodies (10 μg/ml) LM 609 (anti-αvβ3), P1F6 (anti-αvβ5), or P4C10 (anti-β1) were allowed to bind the immobilized anti-mouse antibody for 14 h at 4°C. Subconfluent (~ 50%) HUVEC cells were harvested with EDTA, washed, and resuspended in 20 mM Hepes, 140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$, 5 mM glucose, and 5 mM sodium pyruvate, pH 7.4 (adhesion buffer). Cells were then allowed to attach to antibody-coated dishes for 4 h at 37°C. To maintain cells in suspension, some dishes were coated only with 1% heat-denatured BSA.

Chick CAM angiogenesis assay. 10-d-old chick embryos, purchased from McIntyre Poultry (Lakeside, CA), were incubated at 37°C with 60% humidity. Filter discs (Whatman Inc., Clifton, NJ), 1 × 1 cm, were saturated with 1 μg/ml bFGF (Genzyme Corp., Cambridge, MA) and placed on top of an avascular zone of the CAM as previously described (3). After 20–24 h, at the peak of αvβ3 expression (S. Strömblad, and D.A. Cheresh, unpublished results), embryos were injected intravenously with 100 μl PBS containing antagonists to αvβ3, 300 μg mAb LM 609 or 100 μg peptide 66203 (cyclo-RGDfV). Control embryos received 300 μg mAb CSAT (anti-β1) or 100 μg peptide 69601 (cyclo-RADfV). 48 h later, CAM tissues directly underlying the filters were resected for isolation of nucleic acids or protein. Chick thymus was isolated from 8-wk-old chickens and used as positive control tissue.

Isolation of CAM cells and sorting of endothelial cells. After induction of angiogenesis, CAMs were resected and washed three times in sterile PBS, finely minced, and resuspended in 0.25% bacterial collagenase (Worthington Biochemical Corp., Freehold, NJ). After incubation for 120 min at 37°C with occasional vortexing, cells were washed three times in PBS with 1% BSA, and then incubated with a 1:100 dilution of antiFactor VIII rabbit polyclonal antibodies 016P (BioGenex Labs., San Ramon, CA) at room temperature for 60 min. After three washes in PBS/1% BSA, cells were incubated with FITC-conjugated goat anti-rabbit antibodies at 1:50 dilution (Biosource International, Camarillo, CA) for 45 min at 25°C. Cells were then washed three times (PBS/1% BSA) and positive staining cells were separated from negative by flow cytometry using a FACStar® cell sorter (Becton Dickinson & Co., Mountain View, CA) with Consort 30 software (Becton Dickinson & Co.). Cells incubated only with the secondary antibody were used to set negative markers and the sortings typically yielded 15–20% positive cells.

Preparation of nucleic acids and mRNA analyses. Total nucleic acids (TNA) were isolated by the method of Durnam and Palmiter (17) using 3–5 pooled CAMs per preparation. The TNA concentrations were measured by spectrophotometry at 260 nm. Chicken bcl-2 mRNA and β-actin mRNA was quantified by solution hybridization where 50,000 cpm of a ^{35}S-CTP-labeled (Amersham Corp., Arlington Heights, IL) probe was hybridized to 1–2 μg (β-actin) or 10–20 μg (bcl-2) of the TNA samples as previously described (18) with reagents from Promega Biotech. Inc. (Madison, WI). The bcl-2 clone used was a 532-bp AvaI-BamHI (from 71 to 603) cDNA fragment of the chicken bcl-2 (19), subcloned into a pGEM™ 4Z riboprobe vector (Promega Biotech. Inc.). β-Actin mRNA was quantified using a 2.2-kb cDNA coding for chicken β-actin (20), subcloned into pGEM™ 3. The hybridization signals were compared with that of a standard curve produced using synthetic mRNA strands complimentary to the probes made from the same cDNAs. Three TNA preparations were analyzed for each group and the values were determined using four incubations for each sample at two different concentrations, to ensure that the analyses were within a linear range.

Western blot analyses. Cells or tissues were lysed in a modified RIPA buffer (150 mM NaCl, 10 mM Tris, pH 7.4, 1 mM EDTA, 1% Triton X-100, 1% deoxycholic acid, 1 mM PMSF, with addition of Complete™ protease inhibitor cocktail [Boeringer Mannheim, Mannheim, Germany]). Protein concentrations were determined by a BCA assay (Pierce Chemical Co., Rockford, IL) using BSA as a standard. Cell lysates from three to four pooled CAMs or from HUVEC cells (30 μg protein) were run in an 8% (p53) or 12% (all others) SDS-PAGE, including Rainbow™ molecular weight size markers (Amersham Corp.) under DTT-reduced (50 mM) or non reduced (chicken bcl-2) conditions. Proteins were then transferred to a nitrocellulose filter by semidry blotting in 25 mM Tris, 200 mM glycine, 1.3 mM SDS, 20% methanol, pH 8.3, at 160 mA for 3 h. The filters were stained with 10% Ponceau S solution (Sigma Chemical Co., St. Louis, MO) for 10 min to verify equal loading and transfer efficiency (data not shown). The filters were then blocked in 10% dry milk for 14 h at 4°C and probed with 1–2 μg/ml antibodies sc 492 (anti-bcl-2), sc 493 (anti-bax) (Santa Cruz Biotech., Santa Cruz, CA), anti-p53 polyclonal antibody-7 (Oncogene Science Inc., Cambridge, MA), or anti-p21^{WAF1}polyclonal antibody-5 (Oncogene Science Inc.) for 60 min at RT. After washes in TBS-T (20 mM Tris, 137 mM NaCl, 0.1% Tween 20), signals were detected by horseradish peroxidase–conjugated secondary goat anti-rabbit antibodies at 1:3,000 dilution (Bio-Rad Laboratories, Richmond, CA) or rabbit anti-sheep at 1:5,000 dilution (Oncogene Science Inc.) for 45 min at RT. After extensive washing in TBS-T, electrochemical luimnicense was developed (Amersham Corp.). Membranes were then exposed to X-omat AR x-ray film (Eastman Kodak Co., Rochester, NY) and quantitative values were determined using a Personal Densitometer (Molecular Dynamics, Inc., Sunnyvale, CA) coupled to ImageQuant software (Molecular Dynamics, Inc.).

Electrophoretic mobility-shift assays (EMSA). Nuclear extracts were isolated from 2×10^5 to 1×10^6 cells by the method of Schreiber et al. (21). Cells were suspended in 400 μl cold buffer A (10 mM Hepes, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF) and allowed to swell on ice for 15 min; after which, 25 μl 10% Nonidet P-40 was added and the tubes extensively vortexed. After centrifugation, the nuclear pellet was resuspended in 80 μl ice cold buffer B (20 mM Hepes, pH 7.9, 0.4 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF) and vigorously rocked at 4°C for 15 min. Nuclear extracts were cleared by centrifugation. Protein content was assayed with Bradford reagent (Bio-Rad Laboratories). ^{32}P-end-labeled double-stranded oligonucleotides containing the following human recognition sequences were used: p53 (5′ CAG GCA TGT CTA CAG GCA AAG GCA TGT CTG 3′) and SP1 (5′ ATT CGA TCG GGG CGG GGC GAG C 3′). An oligonucleotide containing a mutated p53 recognition sequence (5′ CAT CAA TGT CTA CAG GCA AAG GCA TAC GTG 3′) served as a control to measure nonspecific binding of proteins present in the nuclear extracts. Each reaction mixture contained 10 fmol probe and 5 μg protein from nuclear extracts. Binding reactions were conducted in 50 mM Hepes, 30% glycerol, 50 mM KCl, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, and 1 mg poly(dI-dC) in a final volume of 15 μl. These reactions were allowed to occur at 4°C for 20 min. For supershift experiments, nuclear extracts were preincubated with 1 μg of anti-p53 monoclonal antibody 421 (Oncogene Science Inc.) for 20 min at 4°C. Complexes were resolved on 6% nondenaturing polyacrylamide gels in 50 mM Tris, 45 mM borate, and 0.5 mM EDTA, pH 7.4. The samples were then subjected to electrophoresis for 2 h at 10V/cm. Gels were dried and subsequently visualized with a PhosphorImager (Molecular Dynamics, Inc.).

Results

Experiments were designed to investigate the role of integrin αvβ3 in the regulation of vascular cell survival during angiogenesis. Angiogenesis was induced with bFGF on the chick CAM of 10-d-old chick embryos. 1 d later embryos were injected intravenously with anti-αvβ3 monoclonal antibody (mAb LM609) or a control antibody directed to chick β1 integrins (mAb CSAT). Administration of anti-αvβ3 specifically leads to extensive apoptosis among proliferating endothelial cells on these CAMs within 48 h (6). To evaluate whether the apoptosis observed was associated with induction of p53 activity, nuclear extracts prepared from these CAMs were examined for p53 binding activity in a gel mobility shift assay. Administration of a single intravenous dose of anti-αvβ3, sufficient to induce apoptosis after bFGF stimulation (2), caused a marked increase in p53 DNA binding activity as measured by the mobility shift (Fig. 1 *A*). However, CAMs examined from embryos injected with anti-β1 showed negligible apoptosis (2) and p53 activity (Fig. 1 *A*). The induction of p53 activity with anti-αvβ3 was specific since excess unlabeled oligonucleotide effectively competed for this binding activity (Fig. 1 *A*) and a mutant oligonucleotide failed to cause the gel shift (data not shown). Also, injection of anti-αvβ3 produced no change in SP1 binding activity (Fig. 1 *A*).

To determine whether this increased p53 activity was associated with endothelial cells within these tissues, single cell suspensions isolated from CAMs by collagenase treatment were stained for the endothelial cell marker, Factor VIII, and were sorted by flow cytometry. Analysis of nuclear extracts from these CAM-sorted endothelial cells revealed increased p53 binding activity after exposure to both bFGF and anti-αvβ3 while SP1 binding activity remained unchanged (Fig. 1 *B*). In contrast, injection of mAb CSAT had no effect on p53 or SP1

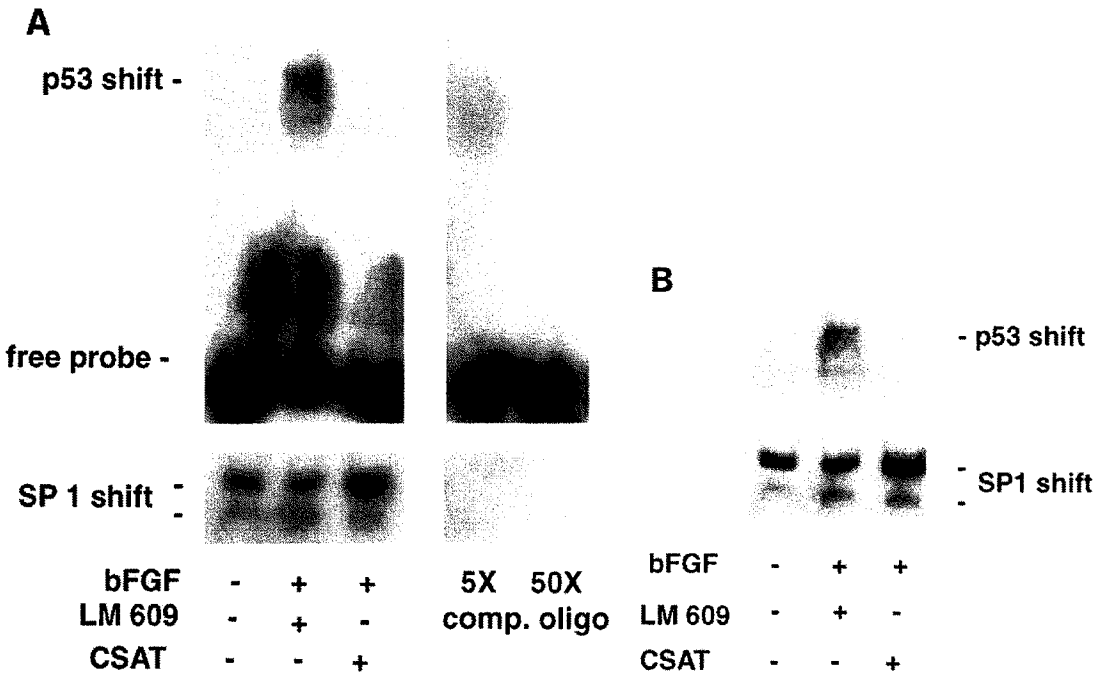
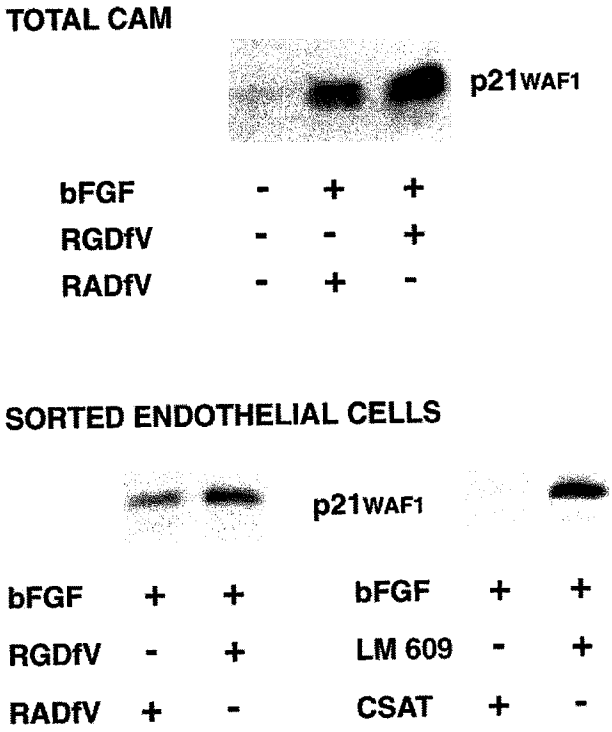


Figure 1. Regulation of p53 activity during bFGF-induced angiogenesis on the chick CAM. (*A*) Angiogenesis was stimulated with bFGF on the chick CAM in the presence of anti–αvβ3 or anti–β1. Nuclear extracts from three pooled CAMs were analyzed by an EMSA, examining p53 or SP1 DNA binding activity as described in Methods (*left*). In parallel, the nuclear extract from the anti–αvβ3 treated CAM was incubated with a 5- or 50-fold excess of unlabeled oligonucleotide (*right*). (*B*) Angiogenesis was stimulated on the CAM with bFGF for 3 d in the presence of anti–αvβ3 or anti–β1. Factor VIII–positive cells (endothelial cells) were then sorted out from a single cell suspension from six to ten pooled CAMs using a FACS® sorter as described in Methods. Nuclear extracts from the sorted endothelial cells were subsequently analyzed by p53 or SP1 EMSAs.



activity, consistent with its inability to affect angiogenesis or apoptosis (2). The observed gel shifts for p53 and SP1 were abolished with excess unlabeled oligonucleotide (data not shown). These findings demonstrate that administration of αvβ3 antagonists in vivo promote apoptosis and p53 activity among angiogenic endothelial cells.

Induction of apoptosis by p53 has been associated with its ability to promote transcription of genes that influence cell cycle progression. For example, p53 is known to induce expression of $p21^{WAF1/CIP1}$, which binds to and inactivates cyclin/Cdk complexes and the proliferating cell nuclear antigen, thereby arresting cells in G1 (22–24). In fact, it was recently shown that overexpression of $p21^{WAF1/CIP1}$ promoted apoptosis of proliferating mammary epithelial cells in vitro, demonstrating a direct role for $p21^{WAF1/CIP1}$ in the apoptosis of proliferative cells (12).

To evaluate whether the induction of p53 activity was associated with increased expression of $p21^{WAF1/CIP1}$, embryos un-

Figure 2. Regulation of $p21^{WAF1/CIP1}$ during bFGF-induced angiogenesis. Angiogenesis was stimulated on the CAM with bFGF for 3 d in the presence of anti–αvβ3 antibodies, αvβ3 antagonist cyclic peptide RGDfV, anti–β1 antibodies or control cyclic peptide RADfV. Lysates from six to ten pooled CAMs (15 μg protein) (*top*) or endothelial cells derived from these CAMs by FACS sorting (8 μg protein) (*bottom*) were analyzed for $p21^{WAF1/CIP1}$ protein levels by Western blotting as described in Methods.

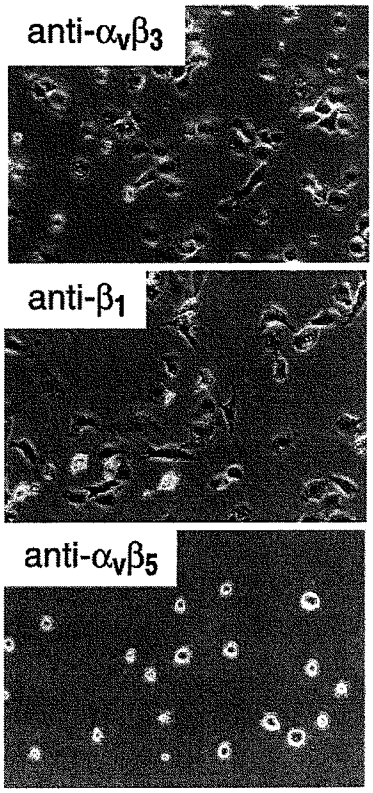


Figure 3. Endothelial cell attachment to immobilized antibodies. HUVECs were plated under serum free conditions on immobilized mAbs LM 609 (anti-αvβ3), P4C10 (anti-β1), or PIF6 (anti-avβ5) as described in Methods. Cells were photographed (100×) after 4 h of attachment.

dergoing angiogenesis in response to bFGF were injected with antagonists of αvβ3. As shown in Fig. 2, administration of antibody or cyclic peptide antagonists of αvβ3 during angiogenesis caused a marked increase in $p21^{WAF1/CIP1}$ protein expression associated with CAM endothelial cells (Fig. 2). These findings suggest that during bFGF-induced angiogenesis in vivo, proliferating endothelial cells depend on a distinct integrin-mediated survival signal associated with inhibition of p53 activity and decreased expression of the cell cycle suppressor $p21^{WAF1/CIP1}$.

To examine whether ligation of αvβ3 is sufficient to suppress p53 activity, human umbilical vein endothelial cells were plated in serum-free conditions on a substrate consisting of immobilized antiintegrin antibodies. When these antibodies are immobilized on a substrate, they are able to mediate integrin clustering at sites of focal adhesion, thereby acting as agonists, as previously described (25). This is opposed to the function of the same antibodies when used in solution or in soluble form in vivo, where they function as antagonists, blocking the integrin function (2, 3). As shown in Fig. 3, endothelial cells attach and spread on immobilized anti-αvβ3 or anti-β1, and attach yet fail to spread on anti-αvβ5. We then examined nuclear extracts of these adherent endothelial cells for p53 DNA binding activity. As expected, there was little if any detectable p53 activity among adherent, proliferative cells maintained in serum containing growth medium (data not shown). However, when cells were plated on the nonadhesive substrate BSA under serum-free conditions, p53 activity was induced (Fig. 4 *A*) consistent with the induction of apoptosis among nonadherent cells (26–30). Interestingly, p53 activity was also induced in cells attached to either immobilized anti-β1 or anti-avβ5 (Fig. 4 *A*). In contrast, cells attached to immobilized anti-αvβ3 showed negligible p53 activity, suggesting this particular adhesion event suppressed p53 activity. These results support the in vivo findings that integrin αvβ3 ligation specifically influences the functional activity of p53. Importantly, regulation of p53 was independent of cell shape and actin assembly since immobilized anti-β1 caused cell spreading yet failed to influence p53 activity. Once again, the specificity of the p53 gel shift was shown by the failure of a mutant oligonucleotide to induce a shift (data not shown), inhibition with excess unlabeled oligonucleotide, and the fact that SP1-binding activity was identical on all substrates (Fig. 4 *A*). In addition, the p53 activity was identified in an antibody-mediated supershift assay (Fig. 4 *B*). Surprisingly, the observed change in p53 DNA binding activity could not be explained by an altered level of p53 protein in these cells since p53 protein levels remained constant under all adhesion conditions (Fig. 4 *B*). These findings demonstrate that ligation of endothelial cell αvβ3 is sufficient to inhibit p53 activity without influencing its expression. Furthermore, in support of the in vivo findings, αvβ3 ligation promotes decreased $p21^{WAF1/CIP1}$ levels in these cells, revealing the association between this cell cycle suppressor and the activity of p53 during αvβ3-mediated adhesion of endothelial cells (Fig. 5). Thus, the ligation state of endothelial cell αvβ3 regulates p53 activity and the expression of $p21^{WAF1/CIP1}$ both in vitro and in vivo, which may account for the role of αvβ3 in vascular cell survival (2).

The role of p53 in promoting apoptosis has also been linked to its ability to repress transcription of bcl-2 while activating that of bax (14–16). Bcl-2 is known to potentiate cell survival based on its ability to dimerize with the death-promoting molecule bax (31–32). Therefore, cell survival is favored by a high bcl-2/bax ratio. To examine the role of endothelial cell αvβ3 ligation on the bcl-2/bax ratio, cells were allowed to attach to immobilized antiintegrin antibodies as described above. While αvβ3 and β1 integrin ligation caused increased bcl-2 expression relative to nonadherent cells, only αvβ3 produced a concomitant decrease in bax expression, resulting in a sharp increase in the bcl-2/bax ratio (Fig. 6). These findings provide further support that ligation of endothelial cell αvβ3 provides a distinct adhesion-dependent survival signal and supports our in vivo observations that antagonists of this integrin selectively promote endothelial cell apoptosis during angiogenesis. Consistent with this hypothesis is the fact that administration of antibody or peptide antagonists of αvβ3 during chick CAM angiogenesis blocks bFGF-induced bcl-2 mRNA and protein expression on the CAM as measured by solution hybridization and Western blot analyses (Fig. 7).

Discussion

Integrin-mediated cell attachment regulates cell survival and proliferation in several cell types in vitro (2, 5, 6, 12, 26–30). Also, cell attachment to extracellular matrix proteins in vitro is capable of regulating the apoptosis-related genes interleukin-1-β–converting enzyme and bcl-2 (29, 30), as well as the cell cycle inhibitors $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ (33). However, in vivo cells typically use multiple integrins for attachment to a wide variety of adhesive proteins. Thus, it remains unclear as to how individual matrix proteins or integrin receptors impact the cell survival within the context of a physiologically relevant extracellular matrix. To this end, we observed that integrin αvβ3, whose expression is enhanced on angiogenic vascular cells, promotes a survival signal since antagonists of this integrin

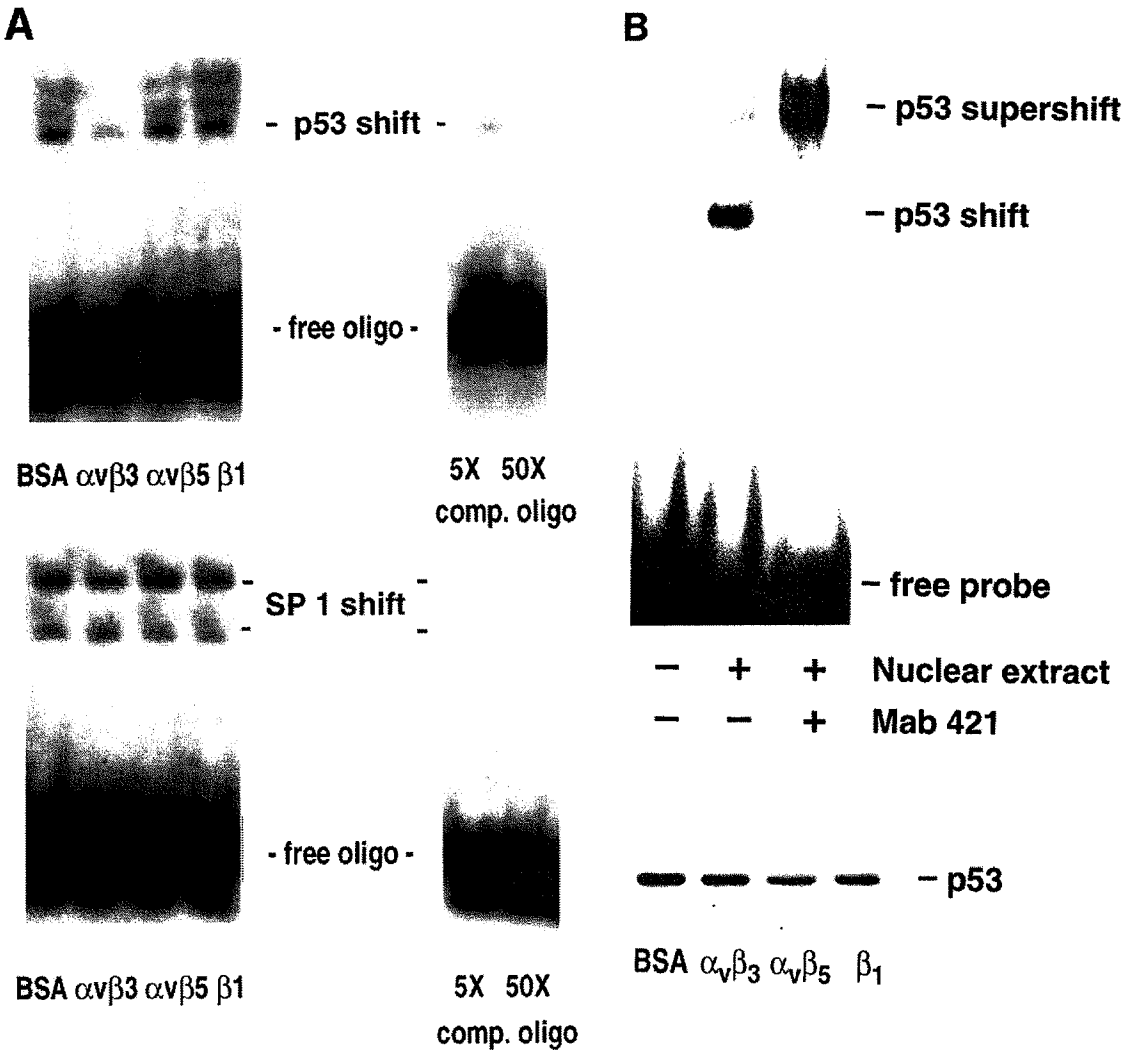


Figure 4. Integrin regulation of endothelial cell p53 activity. (*A*) Nuclear extracts were prepared from HUVECs attached to immobilized mAbs LM 609 (anti–αvβ3), P4C10 (anti–β1), or PIF6 (anti–αvβ3) or maintained in suspension by BSA blocking for 4 h. Nuclear extracts were then used in p53 or SP1 EMSAs as described in Methods (*left*). HUVECs grown in serum-containing media were used as control. Extracts from HUVECs denied attachment were incubated with the ^{32}P-labeled probe in the presence of 5- or 50-fold excess of unlabeled oligonucleotide (*right*). (*B*) (*Top*) Nuclear extract prepared from HUVECs that were prevented from adhering with heat-denatured BSA was analyzed in a super shift gel assay after preincubation in the presence or absence of anti–p53 mAb 421. (*Bottom*) Lysates (15 μg) from HUVECs attached to various immobilized antiintegrin antibodies were analyzed for p53 protein levels by Western blot analysis as described in Methods.

cause unscheduled apoptosis of newly forming blood vessels (2). This results in the disruption of ongoing angiogenesis or neovascularization in the quail embryo, chick CAM, rabbit eye, or human skin transplants on the SCID mouse (2–4, 34, 35). In fact, this leads to either prevention of human tumor growth or regression of preexisting tumors in the chick CAM or in human skin transplanted on SCID-mice (2, 4).

Integrin αvβ3 can recognize several extracellular matrix proteins, including vitronectin, fibronectin, osteopontin, von Willebrand factor, fibrinogen, and proteolyzed collagen (27, 36, 37). At present, it remains unclear as to which of the αvβ3-directed ligand(s) promotes vascular cell survival during angiogenesis. However, recent work in our laboratory demonstrates that the matrix metalloproteinase-2 directly binds to αvβ3 and thereby associates with the surface of angiogenic vascular cells (38). Thus, matrix metalloproteinase-2 once bound to αvβ3, may serve to promote endothelial cell invasion while providing a survival factor, i.e., proteolyzed collagen which binds to αvβ3, thereby facilitating angiogenesis. In support of this contention, αvβ3 recognition of proteolyzed collagen has been shown to promote survival of melanoma cells in vitro (27).

Based on the fact that apoptosis was induced selectively in proliferating vascular cells upon treatment with antagonists to αvβ3 (2), we hypothesized that ligation of αvβ3 during angiogenesis was required for normal cell cycle progression. We found that ligation of αvβ3 during angiogenesis suppresses the expression and/or activity of p53 and the p53-inducible cell cycle inhibitor $p21^{WAF1/CIP1}$. Presence of either p53 or $p21^{WAF1/CIP1}$ in proliferating cells has been found to induce apoptosis (7–11, 12). Thus, it appears that molecules that can promote cell cycle arrest cause apoptosis under other circumstances. The induction of apoptosis during proliferation is presumably caused by

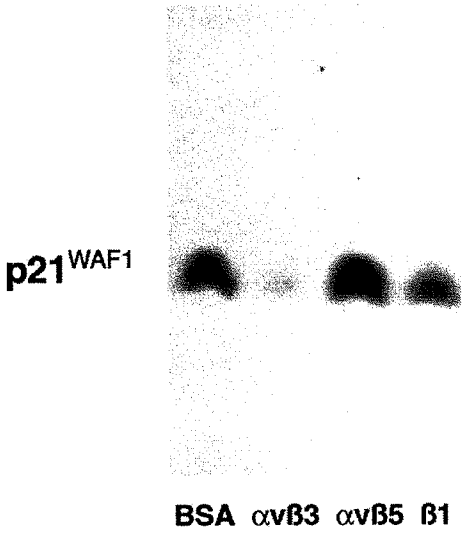


Figure 5. Integrin regulation of $p21^{WAF1/CIP1}$ in endothelial cells. HUVECs were plated under serum free conditions on immobilized mAbs LM 609 (anti–αvβ3), P4C10 (anti–β1), or PIF6 (anti–avβ5) or were denied attachment on a BSA-coated surface for 4 h. Cells were then collected and lysates were analyzed for $p21^{WAF1/CIP1}$ protein levels by Western blotting as described in Methods.

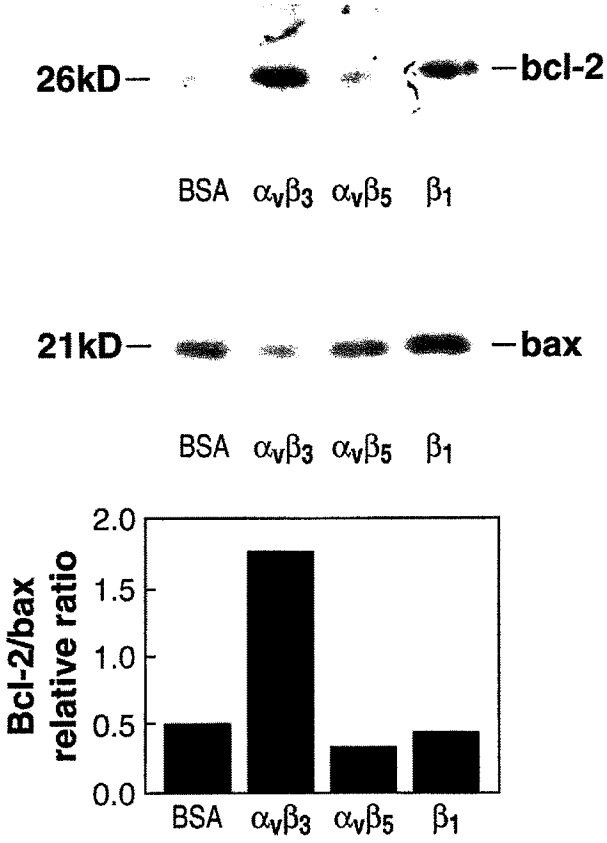


Figure 6. Integrin-dependent regulation of endothelial cell bcl-2 and bax protein levels. HUVECs were plated under serum free conditions on immobilized mAbs LM 609 (anti–αvβ3), P4C10 (anti–β1), or PIF6 (anti–αvβ3) or were denied attachment on a BSA-coated surface for 4 h. Cells were then collected and lysates were analyzed for Bcl-2 and bax protein levels by Western blotting as described in Methods. The bcl-2 and bax protein levels analyzed as above were then quantified by densitometry. The bars represent the relative ratio based upon quantities of bcl-2 and bax as measured by densitometry analysis of autoradiographs and do not represent an absolute molar ratio.

incompatible molecular signals when stimulation of DNA synthesis occurs concurrent with growth arrest signals.

Furthermore, antagonists of αvβ3 administered during angiogenesis specifically prevented an increase in the bcl-2 levels, consistent with the role of p53 as a transcriptional repressor of bcl-2 (14, 15) and with the induction of vascular cell apoptosis. This is consistent with previous studies showing that lack of attachment to the extracellular matrix results in apoptosis of various cell types (2, 12, 26–30, 39, 40). It has been suggested that this phenomenon is caused by the lack of attachment per se (28), but other studies, including data presented here, clearly point out that cell survival is mediated through distinct signals from specific integrins and in some cases also requires a three-dimensional matrix (2, 12, 27, 29, 30). However, various integrins can serve to promote cell survival. For example, distinct β1-integrins can mediate cell survival in mammary cells and chinese hamster ovary cells in vitro, whereas αvβ3 can mediate melanoma cell survival in three-dimensional collagen and survival of proliferating vascular cells in vivo (2, 12, 27, 29, 30). The integrin required for cell survival and the integrin-dependent survival signals might thus be cell type and condition specific.

While the use of receptor antagonists in vivo can suggest receptor function, we performed experiments to directly examine the effect of αvβ3 ligation on cultured human endothelial cells. In this case, antiintegrin antibodies were immobilized on a substrate facilitating their use as agonists of integrin function, thereby promoting adhesion of cells via clustering of a given integrin. This approach has been successfully used to examine integrin-specific signaling events in the absence of growth factors and other adhesion events, also demonstrating that distinct integrins mediate different signals (25, 41, 42).

Inhibition of intercellular contact in a colon carcinoma cell line in vitro, mediated by an undefined αv integrin, induces relocalization of p53 to the nucleus (39). However, it is unclear if this translocation is associated with any change in p53 DNA binding activity. We here show that αvβ3-mediated endothelial cell adhesion selectively reduces p53 activity and $p21^{WAF1/CIP1}$ levels, since attachment via anti–β1 integrins or αvβ5 does not. Furthermore, the degree of cell spreading has been suggested as an important factor for endothelial cell survival (40). However, we demonstrate that attachment to either anti–αvβ3 or anti–β1 resulted in equivalent cell spreading, yet these substrates differed in their ability to regulate p53 activity and $p21^{WAF1/CIP1}$ expression. Ligation of αvβ3 also resulted in a sharp increase in the bcl-2/bax ratio, consistent with the role of p53 as a transcriptional repressor of bcl-2 and an activator of bax (14–16). Based on the fact that bcl-2 potentiates cell survival by forming heterodimers with the death promoting molecule bax (31, 32), the increased bcl-2/bax ratio is expected to directly promote endothelial cell survival.

These studies provide a mechanism explaining how antagonists of αvβ3 can block angiogenesis and thereby cause regression of human tumor growth (2, 4). Experiments reported here demonstrate that αvβ3 ligation induces a distinct endothelial

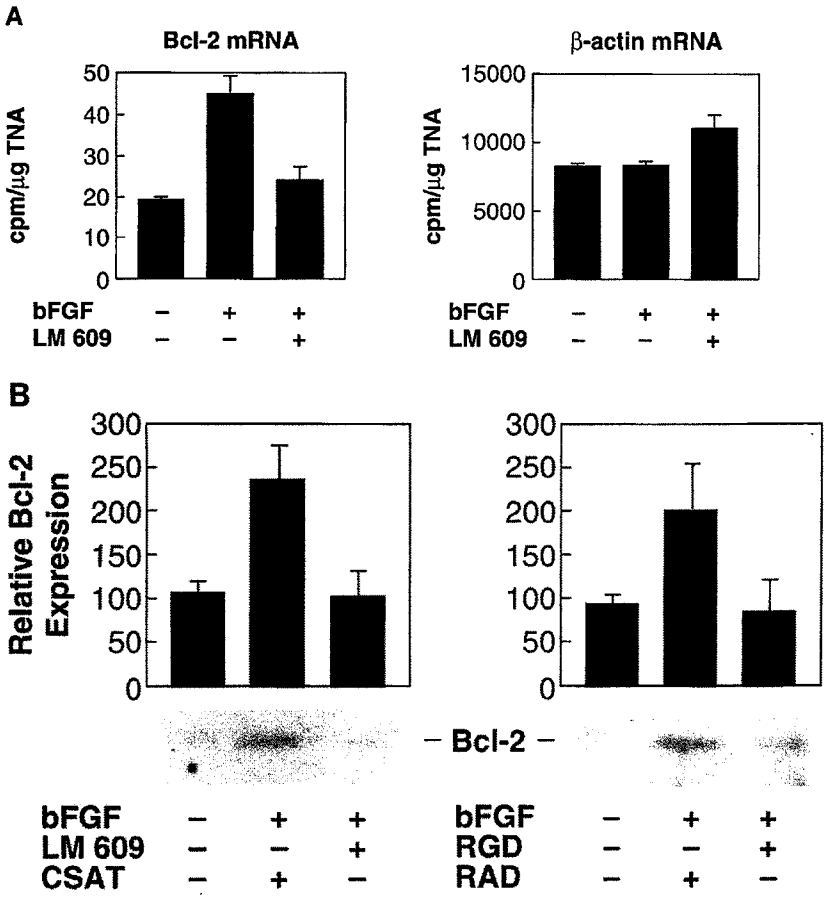


Figure 7. Regulation of bcl-2 during chick CAM angiogenesis. (*A*) Angiogenesis was stimulated by bFGF in the chick CAM for 3 d in the presence or absence of mAb LM609 (anti–αvβ3) and bcl-2 mRNA and β-actin mRNA levels were measured by solution hybridization as described in Methods. Each bar represents the mean of three TNA preparations±SD. Each preparation was from four to five pooled CAM tissues. (*B*) Angiogenesis was stimulated as above in the presence of mAb LM 609 (anti–αvβ3) or mAb CSAT (anti-β1) (*left*) or in the presence of cyclic RGD (anti–αvβ3) or RAD (control) peptides (*right*). CAM tissues were collected and cell lysates of four to five pooled CAMs were analyzed for bcl-2 protein levels by Western blotting under nonreducing conditions as described in Methods. The intensity of the band representing bcl-2 (indicated) was then quantified by densitometry (*bars*). Bars represent the mean values of three separate experiments±standard error.

cell survival signal in vitro and in vivo. Importantly, during bFGF- and tumor-induced angiogenesis, antagonists of this integrin block angiogenesis by promoting unscheduled apoptosis of newly growing blood vessels. Our findings provide a link between αvβ3 ligation, p53 activity, expression of the cell cycle inhibitor $p21^{WAF1/CIP1}$, and vascular cell survival. Thus, during angiogenesis, ligation of endothelial cell αvβ3 is required for the suppression of apoptosis and of conflicting growth arrest signals, thereby facilitating the proliferation and maturation of new blood vessels.

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APPENDIX B

Accelerated Publication

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Loss of p53 Compensates for α_v-Integrin Function in Retinal Neovascularization*

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α_v-Integrin antagonists block neovascularization in various species, whereas 20% of α_v-integrin null mice are born with many normal looking blood vessels. Given that blockade of α_v-integrins during angiogenesis induces p53 activity, we utilized p53 null mice to elucidate whether loss of p53 can compensate for α_v-integrin function in neovascularization of the retina. Murine retinal vascularization was inhibited by systemic administration of an α_v-integrin antagonist. In contrast, mice lacking p53 were refractory to this treatment, indicating that neovascularization in normal mice depends on α_v-integrin-mediated suppression of p53. Blockade of α_v-integrins during neovascularization resulted in an induction of $p21^{CIP1}$ in wild type and, surprisingly, in p53 null retinas, indicating that α_v-integrin ligation regulates $p21^{CIP1}$ levels in a p53-independent manner. In conclusion, we demonstrate for the first time an *in vivo* intracellular mechanism for compensation of integrin function and that p53 and α_v-integrins act in concert during retinal neovascularization.

We previously found that integrin $\alpha_v\beta_3$ is preferentially expressed on newly forming blood vessels and is functionally involved in controlling angiogenesis stimulated by basic fibroblast growth factor or tumor necrosis factor-α, whereas another α_v-integrin, $\alpha_v\beta_5$, is functional in vessel formation induced by vascular endothelial growth factor or transforming growth factor-α (1, 2). Importantly, antagonists of α_v-integrins block neovascularization in various animal models with or without exogenous angiogenic stimulation, including chick chorioallantois, in mouse retina, and in human skin transplants in SCID mice, causing apoptosis of proliferating, angiogenic vascular cells (3–8). This suggests that during vessel formation, α_v-integrins promote signaling events ultimately promoting vascular cell survival, thereby facilitating neovascularization. However, whereas 80% of α_v-integrin null mice die in mid-gestation, 20% of these mice survive until 1 day after birth (9). Similarly, combinatorial gene knockout of integrin β_3 and β_5 subunits in mice results in enhanced angiogenesis under certain conditions (10). This indicates either that mice lacking integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ could compensate for the function of α_v-integrins in blood vessel formation or that a function of vascular α_v-integrins, once expressed but blocked or unligated, is to inhibit neovascularization. However, at present it is not known whether possible compensatory or redundant mechanisms can mediate blood vessel formation in the absence of functional α_v-integrins.

EXPERIMENTAL PROCEDURES

$p53^{+/-}$ mice (11) were used to set up $p53^{+/-} \times p53^{-/-}$ breeding pairs. Littermates from such matings were used in the neovascularization assay, and genomic DNA from mouse tails was genotyped for p53 by a 3-primer assay as described previously (12). Newborn mice were injected subcutaneously twice daily, starting within 8 h after birth with 40 μg of cyclo-RGDfV (α_v antagonist peptide 66203) or cyclo-RADfV (control peptide 69601) (lowercase denotes D-amino acids) dissolved in phosphate-buffered saline, pH 7.4. The cyclo-RGDfV peptide binds specifically with high affinity to $\alpha_v\beta_3$- and $\alpha_v\beta_5$-integrins and blocks their function both *in vitro* and *in vivo*, whereas the cyclo-RADfV peptide is non-functional (3, 7, 8, 13). After 2–3 days, the eye globes were taken out, fixed in cold methanol for 10 min followed by 6 min in 4% paraformaldehyde in phosphate-buffered saline, pH 7.4, dissected, stained for collagen type IV, and photographed as previously described (7). The distance from the head of the optic nerve to the edge of the retinal vasculature at 6–8 different representative points was measured for each retina on the photographs, and the mean vascular radius was calculated. The mean vascular area between the two retinas in each animal was then calculated, assuming a circular shape of the vasculature. Without treatment, no difference in vascular areas was observed between p53 heterozygous and p53 null mice, and therefore, to standardize and compare the results from different litters, the mean retinal vascular area in p53 null mice in each litter was considered to represent a fully developed vasculature. All p53 genotyping and measurements of the retinal vasculature were performed in a double-blind fashion to avoid any bias. For measurements of wild type retinas, the mean vascular area of control treated retinas was considered as fully developed and compared with anti-α_v-treated retinas within the same litters. For Western blot analysis, retinas were fixed only in cold methanol and dissected. Retinas were then lysed in a modified radioimmune precipitation buffer and analyzed by Western blot as previously described (5) using 1 μg/ml anti-$p21CIP1^{WAF-1/CIP-1}$ polyclonal antibodies (ab-5, Oncogene, Cambridge, MA), anti-α_v cytoplasmic tail polyclonal antibodies (Chemicon), or anti-actin monoclonal antibody JLA20 (Developmental Studies Hybridoma Bank, University of Iowa).

RESULTS

Interestingly, inhibition of angiogenesis by blockade of α_v-integrins is accompanied by an induction of endothelial cell p53 activity (5). Based on this, we hypothesized that loss of p53 might compensate for α_v-integrin function during neovascularization. To examine this possibility, we analyzed p53 null mice, where the effect of a specific α_v-integrin antagonist was studied on retinal neovascularization. Mouse retinal neovascularization occurs during the first days after birth, and therefore, newborn mice were treated with an α_v-integrin antagonist as described (7). The retinal vasculature in wild type newborn

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mice treated for 3 days with the α_v-antagonist was significantly less developed than retinas from control treated mice (Table I). When accounting for the retinal vascular area already existing when the treatment started, the inhibition was close to 100%, in accordance with our previous observations after 4 days of treatment (7). Targeted p53 null males were then mated with p53 heterozygous females. This type of mating was not only necessary for sufficient embryonic survival but also allowed for a comparison of p53 null mice with heterozygous mice within the same litter in a double-blind fashion, including animals of exactly the same age receiving identical treatment. No difference in retinal vascularization could be observed between p53 null and heterozygous mice during the first 3 days (data not shown). In addition, no difference in degree of neovascularization between p53 null and heterozygous mice could be observed after control treatment (Fig. 1*B* and Table I). This indicates that p53 does not influence normal vascularization of the retina. We then treated entire litters of newborn mice of a mixed genotype (see above) with the α_v-integrin antagonist. Interestingly, p53 heterozygous animals had a markedly less developed retinal vasculature compared with p53 null mice when treated with the α_v-antagonist (Fig. 1 and Table I). Statistical analysis of measurements performed on the vascular area of these retinas revealed that the vascular development in anti-α_v-treated p53 heterozygous was suppressed, a suppression that was found to be statistically significant ($p = 5.7 \times 10^{-7}$) when compared with p53 null animals in the same litters receiving identical treatment (Table I). This result closely resembled the difference seen between retinas from antagonist *versus* control treated wild type mice (Table I). Taken together, these findings indicate that although p53 expression does not influence normal neovascularization, loss of p53 compensates for the function of α_v-integrins in neovascularization.

TABLE I
Statistical evaluation of the effect on retinal vascular development by treatment with an α_v-integrin antagonist

Statistical evaluation of measured retinal vascular areas. For $p53^{+/-}$ and $p53^{-/-}$, given p values represent statistical significance for the indicated groups compared to identically treated homozygous ($p53^{-/-}$) mice according to an unpaired two-tail t-test using Microsoft Excel software. For wild type (wt) mice, the given p value represents statistical significance for treated *versus* control-treated retinas within the same litters, analyzed by an unpaired t-test. All measurements and genotyping were performed in a double-blind fashion.

Treatment	p53 genotype	% undeveloped vasculature (± S.D.)	n	p value (t-test)
Control peptide	−/−	0 ± 28	9	
	+/−	−5 ± 27	13	0.68
α_v antagonist peptide	−/−	0 ± 14	14	5.7×10^{-7}
	+/−	34 ± 15	17	
Control peptide	wt	0 ± 7.4	8	
α_v antagonist	wt	42 ± 9.4	8	1.8×10^{-9}

A possible explanation for the lack of response to the α_v-integrin antagonist in p53 null mice could be deficient retinal integrin α_v expression. To test this possibility, retinal lysates were analyzed for α_v protein levels by Western blot analysis. As shown in Fig. 2*A*, α_v-integrin levels in p53 null retinas do not differ from that in p53 heterozygous mice. Another possibility for a lack of response to the α_v antagonist in p53 null mice could be that cells lacking p53 are insensitive to this treatment because of alterations in α_v-integrin function at the cell surface. To examine this possibility, we examined α_v-integrin function of mouse embryonic fibroblasts lacking p53, including their responsiveness to the α_v-integrin antagonist and compared them to mouse fibroblasts expressing p53. As shown in Fig. 2*B*, the responsiveness to the antagonist in inhibiting α_v-dependent attachment to vitronectin was virtually identical in p53 null fibroblasts and the mouse fibroblast cell line NIH 3T3, demonstrating that lack of p53 does not cause a general insensitivity to α_v-integrin antagonists. These findings reveal that loss of p53 does not affect expression levels or general function of α_v-integrins. Instead, we conclude that intracellular events involving p53 mediate the inhibition of neovascularization by α_v antagonists, events that may be related to the activation of endothelial cell p53 that we previously observed upon α_v-integrin blockage during angiogenesis (5).

p53 is a known activator of the cell cycle suppressor $p21^{CIP1}$. In fact, in addition to regulating p53, ligation of integrin $\alpha_v\beta_3$ in endothelial cells also suppresses $p21^{CIP1}$ protein levels during angiogenesis (5). In UV-irradiated fibroblasts, p53 exerts its functional effect on cell cycle arrest by transcriptional activation of the Cdk inhibitor $p21^{CIP1}$ (14). As shown in Fig. 2*C*,

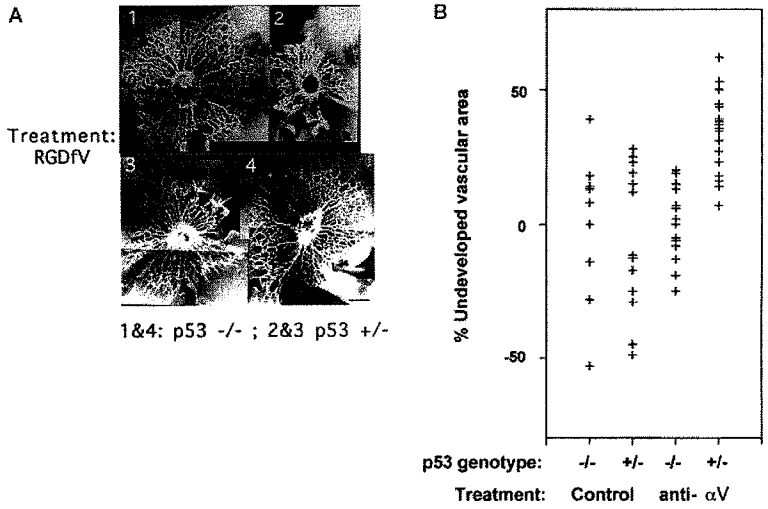

FIG. 1. **Retinal neovascularization in genetically targeted p53 null mice is refractory to systemic treatment with an integrin α_v antagonist.** Newborn mice were injected subcutaneously twice daily with an integrin α_v antagonistic or control cyclic peptides for 2–3 days. Retinas were dissected, stained for collagen type IV (vessel basement membrane), mounted flat, and photographed (10×) as described under "Experimental Procedures." *A*, representative retinas from newborn mice of a mating between a p53 null male and a p53 heterozygous female, where the entire litter was treated for 2½ days with the α_v antagonist. *Size bar* is 150 μm. *B*, measurements of development of the retinal vasculature of 4–5 litters per group were standardized for comparison as described under "Experimental Procedures" and plotted as % undeveloped vasculature. Each point represent the mean retinal vascular area in one mouse calculated as a mean between the two retinas in each animal.

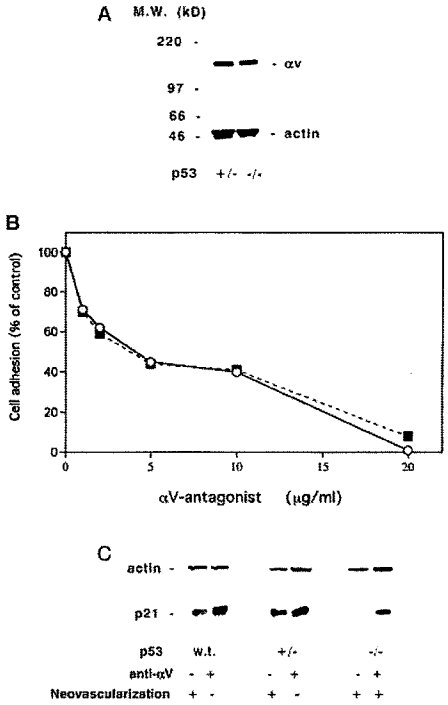

FIG. 2. **Retinal α_v-integrin expression levels, sensitivity to an α_v antagonist, and regulation in the retina of p21^{CIP1} by the α_v antagonist are independent of p53.** *A*, retinas isolated and pooled from three to four p53$^{+/-}$ and p53$^{-/-}$ mice, respectively, were subjected to Western blot analysis for integrin α_v protein expression levels using actin levels as control. *B*, p53$^{-/-}$ (*filled squares*) and NIH3T3 (p53$^{+/+}$) (*open circles*) mouse fibroblasts were analyzed for their sensitivity to the integrin α_v antagonist used in the *in vivo* experiments, here assayed as cell adhesive capacity to vitronectin described previously (23) allowing cells to adhere for 10–15 min. The values are expressed as percent of cell adhesion in the absence of inhibitor and represent mean values between three distinct experiments at each concentration of the α_v antagonist, which in turn was analyzed in triplicate within each experiment. *C*, retinas isolated and pooled from three to four wild type, p53$^{+/-}$ or p53$^{-/-}$ mice, treated with or without α_v antagonist, respectively, were subjected to Western blot analysis for p21^{CIP1} protein levels. Presence or absence of full neovascularization in the respective retinas are indicated as + or −.

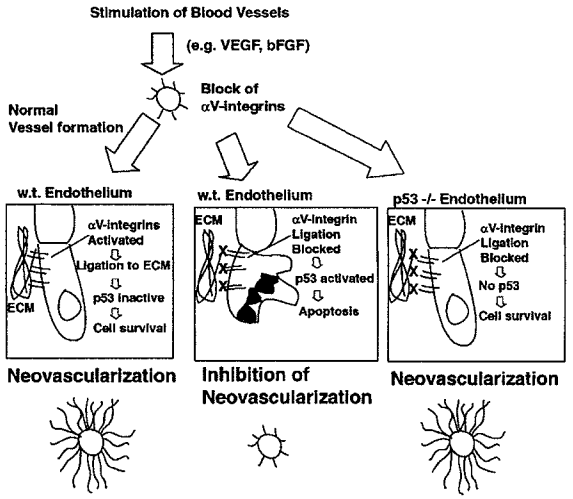

FIG. 3. **Hypothetical model for role of p53 in compensating for α_v-integrin function during blood vessel formation.** In neovascularization of wild type animals (*left*), $\alpha_v\beta_3$-and/or $\alpha_v\beta_5$-integrins are activated (1, 2). The α_v-integrins are then allowed to ligate to their provisional matrix, a ligation that is necessary to keep endothelial cell p53 inactive and cells surviving (vascularization is facilitated (3, 5)). When α_v-integrins are blocked in wild type animals (*middle*) and thereby prevented from forming clusters, endothelial cell p53 activity is induced, and the vascular cells undergo apoptosis leading to a block of blood vessel formation (5). However, when p53 is absent during vascularization, inhibition of α_v-integrins does not affect the formation of viable vessels (*right*). Taken together, this suggests that p53 and α_v-integrins are linked into the same pathway in the control of blood vessel formation. *VEGF*, vascular endothelial growth factor; *bFGF*, basic fibroblast growth factor; *ECM*, extracellular matrix.

blockade of α_v-integrin during neovascularization induces p21^{CIP1} levels in wild type and in p53 heterozygous retinas. Surprisingly, whereas untreated p53 null mice display no detectable p21^{CIP1}, the numeric increase in p21^{CIP1} levels by anti-α_v treatment of these mice is similar to what is observed in wild type mice, resulting in a higher relative increase. This indicates that the regulation of p21^{CIP1} by integrin α_v during neovascularization is independent of p53. Furthermore, the fact that p21^{CIP1} is induced in p53 null retinas upon blockade of α_v-integrins while neovascularization is still active suggests that this induction of p21^{CIP1} is not sufficient to block neovascularization, although we cannot exclude that the somewhat higher total levels of p21^{CIP1} in heterozygous animals might contribute to this blockade.

DISCUSSION

Studies using targeted gene knockout mice have in some cases revealed surprising results in that expected phenotypes were not found. This is particularly surprising for molecules found to play a role in certain *in vivo* events by previous loss of function studies, including for integrin α_v and the capacity for at least 20% of α_v-integrin null mice to form blood vessels (9) and for the capacity of integrin β_3 and β_5 subunit combinatorial gene knockouts to support enhanced pathological angiogenesis (10). In some cases, combinatorial knockout of two or three related genes has demonstrated compensatory mechanisms by displaying phenotypes missing in single gene knockout mice. However, it is unclear as to how the functions of α_v-integrins can be compensated for. To this end, although it does not represent the only possible mechanism of α_v-integrin compensation, our finding that p53 null mice form blood vessels in the absence of functional α_v-integrins that are critical in wild type mice reveals the first *in vivo* example of an intracellular mechanism that is able to compensate for loss of integrin function.

Alternatively, the function of α_v-integrins in neovascularization in wild type animals may be to negatively regulate and balance vessel formation in an unfavorable extracellular matrix environment in order to prevent angiogenesis in inappropriate locations. Such a function of α_v-integrins would then lead to enhanced angiogenesis when $\alpha_v\beta_3$ is lacking as suggested by Reynolds *et al.* (10). In support of this model, caspase-8 was activated at the cell surface in other cell types by unligated integrin $\alpha_v\beta_3$, thereby causing apoptosis (15). This mechanism might be related to p53, because caspase-8 plays a role in certain p53-induced apoptosis (16). Nevertheless, in both alternative models for the function of α_v-integrins, our results suggest that p53 may mediate α_v-integrin regulation of cell survival during neovascularization.

Neovascularization is a critical component of tumor growth, where a tumor is unable to grow beyond a minimal size without new blood vessels (17). In fact, we previously observed that α_v-integrin antagonists could block the growth of human tumors in animal models (3, 4). In addition, uncontrolled ocular neovascularization is a major cause of blindness in various ocular diseases, including diabetic retinopathy, presumed ocular histoplasmosis syndrome, and age-related macular degeneration. Integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ may be involved in the regulation of neovascularization of these diseases as systemic treatment with α_v-integrin antagonists block retinal neovascu-

larization (7, 8). This suggests that α_v-integrin antagonists constitute a potential therapy for ocular diseases and cancer. Our findings indicate that the molecular mechanism for this potential anti-angiogenic treatment actively involves p53, similar to what was recently indicated for angiostatin and TNP-470 (18–20).

We were unable to detect apoptosis in the retinal vasculature because of an obscuring background with a large number of apoptotic cells in the whole mounts of developing retinas with no apparent differences between the groups (data not shown). However, previous studies in other models clearly demonstrate that blocking of α_v-integrins during neovascularization leads to vascular cell apoptosis (3, 5). This suggests that the inhibition of vessel formation by α_v antagonists may be caused by induction of apoptosis of the forming vascular cells, and the fact that vascular formation in p53 null mice is refractory to α_v antagonist treatment suggests that these vessels do not undergo apoptosis (Fig. 3). Whereas p53 may mediate α_v-integrin-regulated apoptosis in vascular cells, induction of p53 by loss of integrin ligation does not constitute a generic mechanism for regulation of cell survival in all cells and by all integrins. For example, ligation of integrin $\alpha_3\beta_1$ in an *in vitro* model of mammary epithelial cells lead to apoptosis only in the absence of functional p53 (21), a mechanism that appears to be the opposite of our findings on vascular cell integrin $\alpha_v\beta_3$ and p53 during neovascularization. In future studies, it will be interesting to elucidate whether downstream integrin signaling pathways such as activation of ERKs[1] might be involved in the regulation of p53, because ERK1/2 signaling was identified as another critical α_v-integrin-mediated event during angiogenesis (22). In addition, it remains to be elucidated whether the p53-mediated response to α_v antagonist treatment in vascular cells is functionally related to activation of caspase-8 by unligated $\alpha_v\beta_3$ (15).

In conclusion, we demonstrate that loss of p53 compensates for the function of α_v-integrins in retinal neovascularization, possibly by interfering with α_v-integrin regulation of vascular cell apoptosis. This indicates a critical function for α_v-integrin ligation during neovascularization in suppressing p53 and that p53 constitutes an important part of the control of neovascularization.

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[1] The abbreviation used is: ERK, extracellular signal-regulated kinase.

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APPENDIX C

Restoration of the tumor suppressor function to mutant p53 by a low-molecular-weight compound

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The tumor suppressor p53 inhibits tumor growth primarily through its ability to induce apoptosis. Mutations in p53 occur in at least 50% of human tumors. We hypothesized that reactivation of mutant p53 in such tumors should trigger massive apoptosis and eliminate the tumor cells. To test this, we screened a library of low-molecular-weight compounds in order to identify compounds that can restore wild-type function to mutant p53. We found one compound capable of inducing apoptosis in human tumor cells through restoration of the transcriptional transactivation function to mutant p53. This molecule, named PRIMA-1, restored sequence-specific DNA binding and the active conformation to mutant p53 proteins *in vitro* and in living cells. PRIMA-1 rescued both DNA contact and structural p53 mutants. *In vivo* studies in mice revealed an antitumor effect with no apparent toxicity. This molecule may serve as a lead compound for the development of anticancer drugs targeting mutant p53.

The tumor suppressor p53 triggers cell-cycle arrest and apoptosis in response to diverse stress stimuli, including DNA damage, oncogene activation and hypoxia[1–4]. At least 50% of human tumors carry mutant p53 (ref. 5). The fact that the specific DNA binding function of p53 is disrupted in most tumor-derived p53 mutants indicates that this function is critical for p53-mediated tumor suppression. p53-dependent apoptosis seems to have a major role for the efficacy of cancer chemotherapy[6], and tumors carrying mutant p53 are often more resistant to chemotherapy than tumors carrying wild-type p53 (ref. 7).

Tumor cells are likely to be particularly sensitive to p53 reactivation. First, tumor cells are sensitized to apoptosis due to oncogene activation[8]. Second, mutant p53 proteins tend to accumulate at high levels in tumor cells, mainly due to failure of mutant p53 to transactivate mouse double minute-2 (MDM2) whose product induces p53 degradation. Therefore, reactivation of abundant mutant p53 may trigger a massive apoptotic response in tumor cells, whereas normal cells that express minute levels of p53 should not be affected.

Various strategies have been designed to restore function to mutant p53 (ref. 9). The introduction of second-site suppressor mutations can at least partially restore specific DNA binding and/or stabilize the folding of the protein[10–12]. Synthetic peptides derived from the p53 C-terminus can restore the specific DNA binding and transactivation function to mutant p53 and induce p53-dependent apoptosis in tumor cells[13–15]. We sought to identify small molecules with similar activity by screening of a chemical library, and present here a novel compound, PRIMA-1, that can restore sequence-specific DNA-binding, wild-type conformation and transcriptional transactivation to mutant p53. PRIMA-1 induced apoptosis in human tumor cells in a p53-dependent manner and suppressed the growth of human tumor xenografts carrying mutant p53.

Identification of PRIMA-1

To identify compounds that could suppress the growth of human tumor cells in a mutant p53–dependent manner, we established an assay based on Saos-2-His-273 cells carrying tetracycline-regulated mutant p53 (Tet-Off). We treated cells with 25 μM of compounds in the presence or absence of doxycycline and monitored cell growth using the WST-1 proliferation reagent. We identified one compound that suppressed the growth of Saos-2-His-273 cells in a mutant p53–dependent manner (Fig. 1*a*). This compound, 2,2-bis(hydroxymethyl)-1-azabicyclo[2,2,2]octan-3-one, was designated PRIMA-1 (p53 reactivation and induction of massive apoptosis) (Fig. 1*b*). In subsequent experiments, we showed that PRIMA-1 could inhibit growth of several other human tumor-cell lines carrying tetracycline-regulated mutant p53, including SKOV-His-175, SKOV-His-273 and H1299-His-175 (Fig. 1*c*). As the effective concentration of PRIMA-1 is proportional to the number of treated cells, we used different concentrations in the assays described below.

We next analyzed the response to PRIMA-1 of a panel of human tumor-cell lines with different p53 status (p53-null, wild-type p53 and mutant p53), representing various tumor types including colon, lung, ovarian and renal carcinoma, and Burkitt lymphoma (see Supplementary Table A on the supplementary information page of *Nature Medicine* online). The

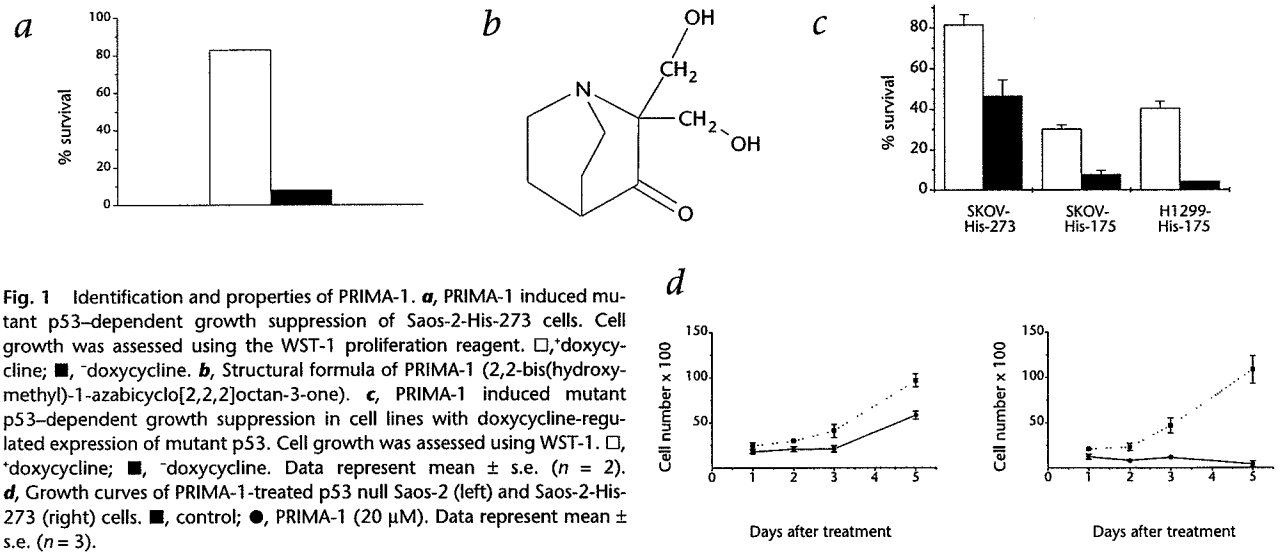


Fig. 1 Identification and properties of PRIMA-1. ***a***, PRIMA-1 induced mutant p53–dependent growth suppression of Saos-2-His-273 cells. Cell growth was assessed using the WST-1 proliferation reagent. □,⁺doxycycline; ■, ⁻doxycycline. ***b***, Structural formula of PRIMA-1 (2,2-bis(hydroxymethyl)-1-azabicyclo[2,2,2]octan-3-one). ***c***, PRIMA-1 induced mutant p53–dependent growth suppression in cell lines with doxycycline-regulated expression of mutant p53. Cell growth was assessed using WST-1. □, ⁺doxycycline; ■, ⁻doxycycline. Data represent mean ± s.e. (n = 2). ***d***, Growth curves of PRIMA-1-treated p53 null Saos-2 (left) and Saos-2-His-273 (right) cells. ■, control; ●, PRIMA-1 (20 μM). Data represent mean ± s.e. (n = 3).

growth suppression effect of different concentrations of PRIMA-1 was assessed using the WST-1 proliferation reagent and compared with that of two anticancer drugs, 5-fluorouracil (5-FU) and doxorubicin. The IC_{50} (concentration of a drug that causes 50% growth inhibition) values for PRIMA-1 varied depending on tumor type. However, comparison between lines of a given tumor type showed that the effect of PRIMA-1 was dependent on mutant p53. In contrast, 5-FU inhibited growth more efficiently in tumor cells carrying wild-type p53 (ref. 18). The effect of doxorubicin was independent of p53 status. Notably, PRIMA-1 had no significant growth-inhibitory effect on non-transformed diploid human fibroblasts.

Analysis of the growth of treated p53 null Saos-2 and Saos-2-His-273 cells over five days showed that PRIMA-1 completely inhibited growth of cells expressing mutant p53, but only caused a minor reduction in growth rate in the absence of mutant p53 expression (Fig. 1*d*).

Restoration of p53-dependent apoptosis

We used FACS and TdT-mediated dUTP nick-end labeling (TUNEL) analyses to determine whether PRIMA-1-induced growth suppression was due to induction of apoptosis. Treatment with 125 μM PRIMA-1 for 48 hours caused a substantial increase in the fraction of cells with a sub-G1 DNA content in the presence of mutant p53 (Fig. 2*a* and *b*), indicating DNA fragmentation and cell death. TUNEL staining of PRIMA-1-treated Saos-2-His-273-cells revealed TUNEL-positive nuclei, indicating cell death by apoptosis (Fig. 2*c*). Moreover, pretreatment with the caspase inhibitor Z-DEVD-FMK reduced PRIMA-1-induced cell death 3-fold and the caspase inhibitor BOC-D-FMK completely abolished PRIMA-1-induced cell death, strongly suggesting that PRIMA-1 triggers cell death by apoptosis.

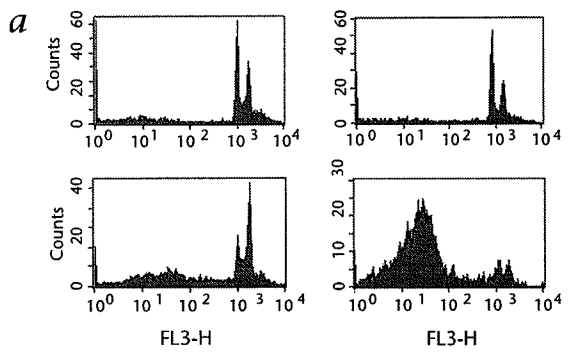
Restoration of wild-type p53 conformation and DNA binding

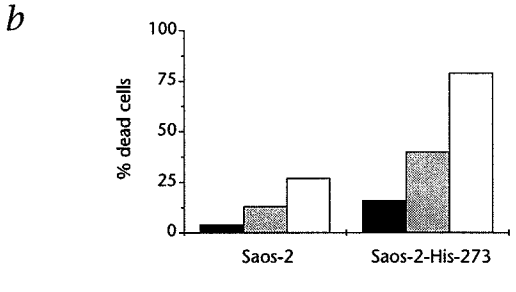
We next investigated whether PRIMA-1 can restore the proper folding of p53, using the conformation-specific monoclonal antibodies PAb1620 and PAb240 in an ELISA. After incubation of recombinant Glutathione-S-Transferase (GST)–wild type p53 protein with PRIMA-1, we observed a 34% increase in the PAb1620-positive fraction accompanied by a 10% decrease in the PAb240-positive fraction (Fig. 3*a*). Moreover, PRIMA-1 preserved the PAb1620 epitope in the recombinant wild-type and His-175 mutant p53 proteins during incubation at 37 °C for 30 minutes (Fig. 3*b*). Importantly, our results demonstrated that PRIMA-1 prevented unfolding of p53 proteins as measured by the appearance of the PAb240 epitope upon heating at 37 °C. The non-conformational DO-1 epitope in the N-terminus of p53 remained unchanged during PRIMA-1 treatment.

Furthermore, we observed a 46% increase in the PAb1620-positive p53 fraction in lysates from SKOV-His-175 cells treated with 150 μM of PRIMA-1 (data not shown). PRIMA-1 treatment of the same cells also caused the appearance of PAb1620-positive immunostaining (Fig. 3*c*). These results demonstrate that PRIMA-1 can restore the wild-type conformation to mutant p53 both *in vitro* and in living cells. Notably, PRIMA-1 treatment of SKOV-His-175 cells resulted in a decrease in total p53 levels according to staining with polyclonal anti-p53 antibodies.

We investigated whether PRIMA-1 could modulate the specific DNA binding of wild-type and mutant p53 proteins. Increasing concentrations of PRIMA-1 were capable of preserving the specific DNA binding of recombinant GST-wild-type p53 during incubation of the protein at 37 °C in a bandshift assay (Fig. 4*a*). PRIMA-1 also reactivated the sequence-specific DNA binding of endogenous His-175 mutant p53 protein in a CW678-cell extract (Fig. 4*b*). Likewise, the sequence-specific DNA binding of endogenous Trp-282 mutant p53 in BL-60 cells was rescued by PRIMA-1 (Fig. 4*c*). The monoclonal antibodies PAb421 and PAb1801 were added to supershift p53–DNA complexes. Neither antibody alone caused any stimulation of specific DNA binding of mutant p53. The position of the wild-type p53–DNA–PAb421 complex is shown in Fig. 4*b* (lane 7).

We further tested the ability of PRIMA-1 to restore the specific DNA binding of a broad series of p53 mutants using cell extracts of human tumor-cell lines as the source of p53. PRIMA-1 restored the specific DNA binding to 13 of 14 p53 mutants tested in bandshift assays (Supplementary Table B). The only exception was the Phe-176 p53 mutant from KRC/Y cells.





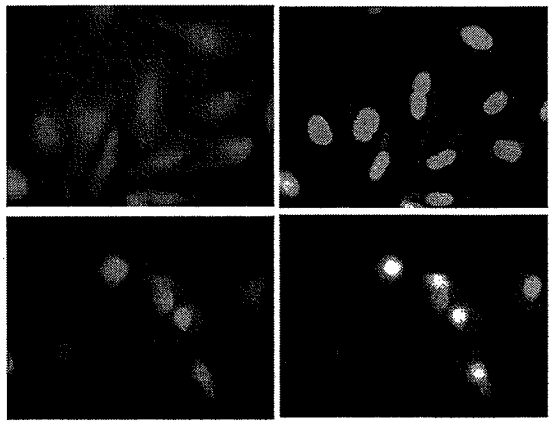


Fig. 2 PRIMA-1 induces cell death by apoptosis. ***a***, FACS profiles of control (top) and PRIMA-1-treated (bottom) Saos-2 (p53-null; left) and Saos-2-His-273 (His-273 mutant p53; right) cells. ***b***, Cell death induced by different concentrations of PRIMA-1 is mutant p53-dependent as shown by FACS analysis. ■, 50 μM; ▒, 75 μM; □, 125 μm. ***c***, TUNEL (left) and Hoeschst (right) staining of control (top) and PRIMA-1-treated (bottom) Saos-2-His-273 cells.

Notably, KRC/Y cells were resistant to PRIMA-1-induced growth suppression (Supplementary Table A).

Transcriptional transactivation restored to mutant p53

We addressed the question whether PRIMA-1 could also restore the transcriptional transactivation function to mutant p53 in living cells. Treatment of A431 cells that carry endogenous His-273 mutant p53 and a stably transfected p53-responsive LacZ reporter with 50 μM PRIMA-1 for 20 hours resulted in the appearance of LacZ-positive cells whereas untreated cells were LacZ-negative (Fig. 5*a*). A strong induction of a p53-responsive enhanced green fluroscent protein (EGFP) reporter was seen in SKOV-His-175 cells expressing mutant p53 after treatment with PRIMA-1 for 24 hours (Fig. 5*b*). In contrast, PRIMA-1-treated SKOV-His-175 cells grown in the presence of doxycycline (p53 off) did not express detectable levels of EGFP.

PRIMA-1 induced the expression of two endogenous p53 target genes, p21 and MDM2, in H1299-His-175 cells expressing mutant p53 and in SW480 colon carcinoma cells expressing endogenous His-273 mutant p53 (Fig. 5*c* and *e*). PRIMA-1 did not cause any induction of MDM2 nor p21 in the absence of mutant p53 expression (Fig. 5*d*). PRIMA-1 did not induce MDM2 nor p21 expression in HCT116 colon carcinoma cells that express wild-type p53 (Fig. 5*e*). 5-FU did not affect p21 nor MDM2 levels in H1299-His-175 (Fig. 5f) and SW480 cells (data not shown).

PRIMA-1-induced apoptosis depends on p53 transactivation

To further ascertain that PRIMA-1 exerts its effect through p53-mediated transcriptional transactivation and *de novo* protein synthesis, we tested the effect of cycloheximide on PRIMA-1-induced growth inhibition. Pretreatment of SKOV-His-175 cells with cycloheximide before addition of PRIMA-1 caused a four-fold increase in cell survival according to the WST-1 proliferation assay. Moreover, SKOV cells carrying tetracycline-regulated His-175-22/23 mutant p53 that has an inactivated transactivation domain were resistant to PRIMA-1 at a range of concentrations, in contrast to SKOV-His-175 cells (Fig. 5*g*). These results indicate that transcriptional transactivation by p53 is critical for PRIMA-1-induced cell death.

Antitumor activity of PRIMA-1 *in vivo*

Intravenous (i.v.) injections of PRIMA-1 in mice did not cause any obvious changes in weight or behavior compared with untreated animals. The average weight of mice treated with PRIMA-1 at the highest dose (100 mg/kg) was 20 g after 1 month of observation, the same as that of control mice. To assess the effect of PRIMA-1 on human tumor xenografts, we inoculated mice with Saos-2-His-273 cells expressing mutant p53. The animals received intratumor (20 mg/kg) or i.v. (20 or 100 mg/kg) injections of PRIMA-1 twice a day for three days. In the control group, the average tumor volume after 59 days was 555.7 mm^3. Mice that received i.v. injections of PRIMA-1 at a dose of 100 mg/kg or 20 mg/kg had an average tumor volume of 11.7 mm^3 and 53 mm^3, respectively (Fig. 6a). Intratumor injections of 20 mg/kg of PRIMA-1 caused a decrease in average tumor volume to 5.3 mm^3. The differences in tumor volumes are statistically significant (P = 0.0026 for intratumor injection, P = 0.003 for i.v. injection). Thus, PRIMA-1 has *in vivo* antitumor activity in this animal tumor model.

To assess *in vivo* reactivation of mutant p53, we examined whether PRIMA-1 could induce p53 target genes in human tumor xenografts. Treatment of SW480 tumor xenografts with PRIMA-1 resulted in induction of both MDM2 and p21 at 16 hours post-treatment (Fig. 6b), in accordance with our *in vitro* results (Fig. 5e).

We next compared the tumor suppressor effect of PRIMA-1 *in vivo* in the presence or absence of mutant p53 expression using mice inoculated with Saos-2 and Saos-2-His-273 cells in the right and left flanks, respectively. We observed a significant decrease in the volumes of Saos-2-His-273 xenografts after PRIMA-1 treatment; the average tumor volume was 48 mm^3 compared to 322 mm^3 in control mice. In contrast, Saos-2 xenografts were not significantly affected; average

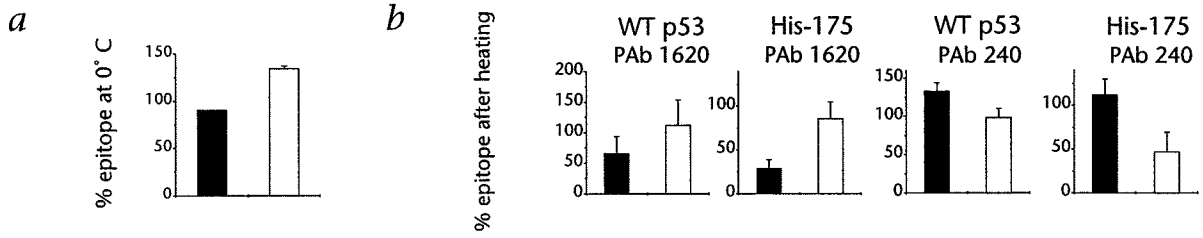
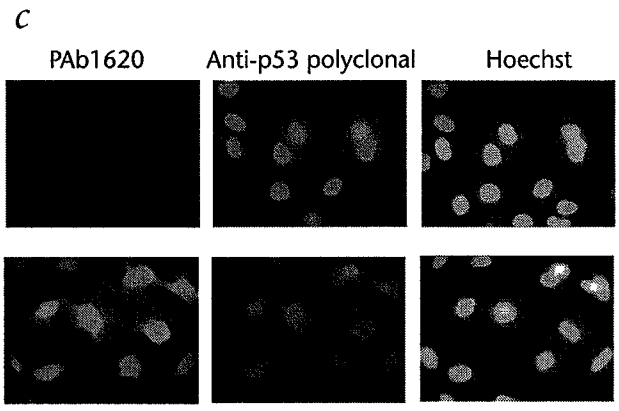
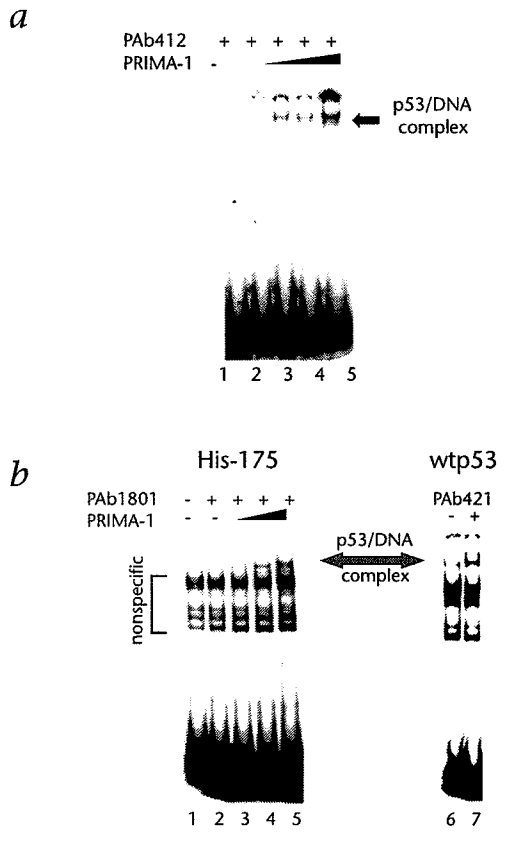


Fig. 3 Effect of PRIMA-1 on the conformation of mutant p53 proteins. ***a***, PRIMA-1 induced the wild-type p53-specific PAb1620 (□) epitope in GST-wild-type p53 protein incubated at 0 °C as detected by ELISA and slight decrease in the PAb240 (■) epitope. ***b***, PRIMA-1 prevented loss of the PAb1620 epitope and gain of the PAb240 epitope upon heating of GST-wild-type p53 and GST-His-175 mutant p53. ■, untreated; □, PRIMA-1 treated. ***c***, PRIMA-1 (100 μM) induced the wild-type p53-specific PAb1620 epitope in SKOV-His-175 cells as shown by PAb1620 immunostaining.



tumor volumes in the PRIMA-1-treated and control mice were 703 mm^3 and 981 mm^3, respectively (Fig. 6*c*). The differences in tumor volumes are statistically significant ($P = 0.008$ for Saos-2-His-273, $P = 0.04$ for Saos-2). Histological examination of tumor sections revealed induction of morphological changes in PRIMA-1-treated Saos-2-His-273 xenografts, including tumor-cell depletion and absence of vascularization, whereas PRIMA-1 did not cause any significant morphological changes in Saos-2 xenografts (Fig. 6*d*). Thus, PRIMA-1 suppressed *in vivo* tumor growth in a mutant p53–dependent manner.



Discussion

Our previous studies provided compelling evidence that the specific DNA binding, transactivation and apoptosis-inducing functions of several mutant p53 proteins could be reactivated by a synthetic peptide derived from the p53 C-terminal domain[13,15]. These findings prompted us to search for even more potent p53 reactivating molecules. We report here the identification of a small molecule, PRIMA-1, that can restore the tumor suppressor function to tumor-derived mutant p53 proteins.

We identified PRIMA-1 by screening a chemical library for compounds that selectively inhibit the growth of tumor cells in the presence of mutant p53 expression. Our approach allowed identification of compounds that can target p53 in the context of multiple cellular proteins. Moreover, compounds were selected for their ability to rescue the growth suppressor function

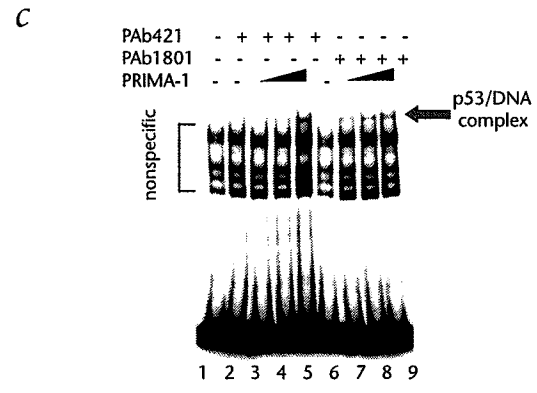


Fig. 4 Effect of PRIMA-1 on the DNA binding of mutant p53 proteins. ***a***, PRIMA-1 preserved the DNA binding activity of GST-wild-type p53 upon heating. ***b***, PRIMA-1 restored DNA binding activity to His-175 mutant p53 in a CW678 cell extract. ***c***, PRIMA-1 restored DNA binding activity to Trp-282 mutant p53 in a BL60 cell extract.

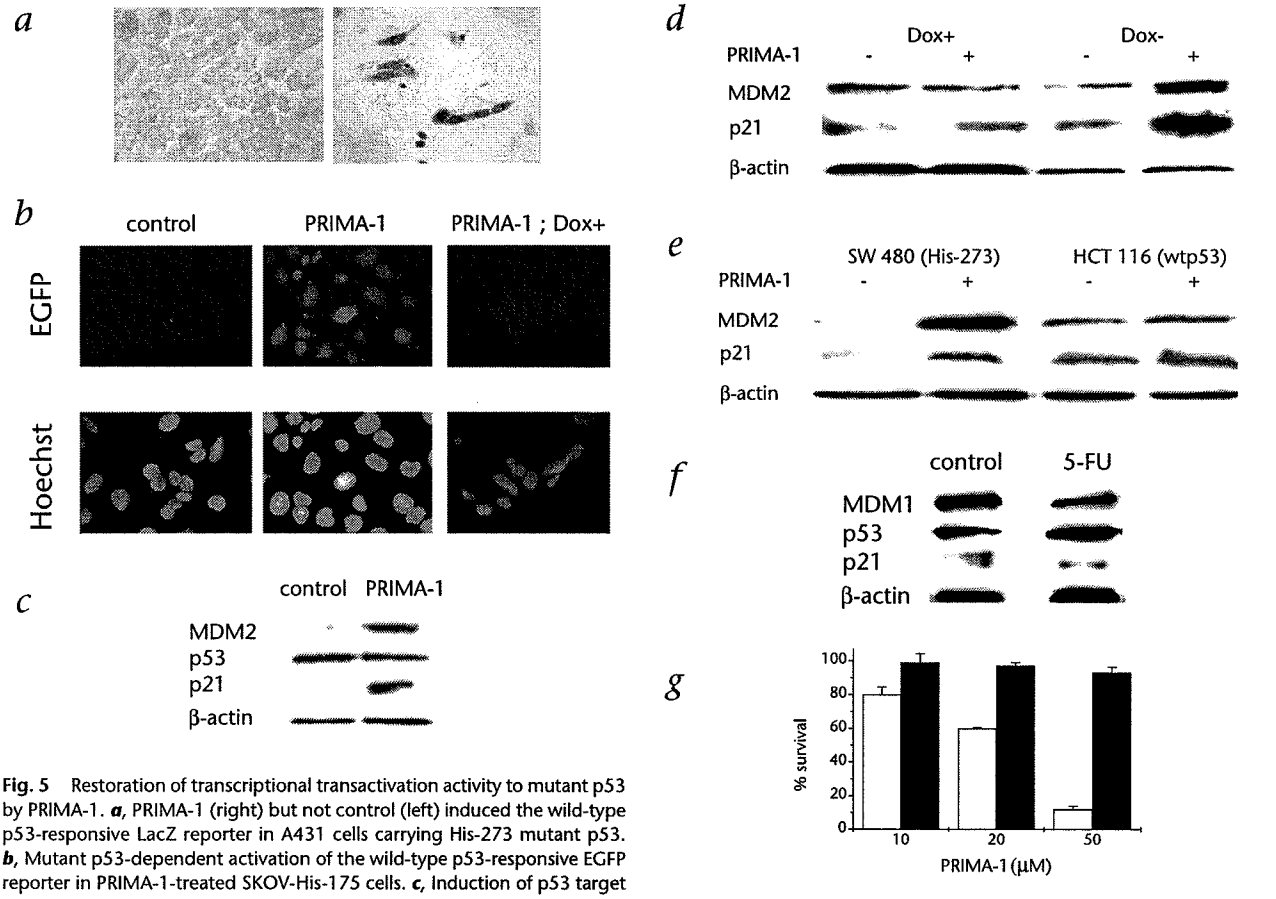


Fig. 5 Restoration of transcriptional transactivation activity to mutant p53 by PRIMA-1. ***a***, PRIMA-1 (right) but not control (left) induced the wild-type p53-responsive LacZ reporter in A431 cells carrying His-273 mutant p53. ***b***, Mutant p53-dependent activation of the wild-type p53-responsive EGFP reporter in PRIMA-1-treated SKOV-His-175 cells. ***c***, Induction of p53 target genes in PRIMA-1-treated H1299-His-175 cells. ***d***, Mutant p53-dependent induction of p53 target genes in H1299-His-175 cells. ***e***, Induction of p53 target genes in PRIMA-1-treated SW480 colon carcinoma cells carrying endogenous His-273/Ser-309 mutant p53. PRIMA-1 did not induce the same p53 target genes in HCT-116 colon carcinoma cells carrying endogenous wild-type p53. ***f***, Treatment with 5-FU did not induce the p53 target genes p21 and MDM2 in H1299-His-175 cells. Protein levels were determined by western-blotting in *c*, *d*, *e* and *f*. ***g***, Effect of PRIMA-1 on SKOV cells carrying the transcriptionally inactive His-175-22/23 mutant p53. ■, SKOV-His-175-22/23; □, SKOV-His-175.

of p53 in living cells. Compounds with nonspecific toxic effects and compounds that do not enter cells should not score in the assay. This screening approach may lead to further identification of compounds that reactivate mutant p53 through previously unknown mechanisms.

We verified the ability of PRIMA-1 to induce apoptosis in a mutant p53–dependent manner using a panel of cell lines with tetracycline-regulated expression of the most common p53 mutant proteins, as well as tumor-cell lines of various origin carrying p53 deletion or different endogenous mutant p53 proteins. The correlation between p53 status and the effect of PRIMA-1 supports the conclusion that PRIMA-1 selectively suppresses tumor-cell growth by inducing apoptosis in cells expressing mutant p53.

Our finding that PRIMA-1 can restore the DNA binding to 13 of 14 p53 mutants tested in bandshift assays corroborates our growth suppression data and demonstrates that both structural and DNA contact mutants can be rescued. It is particularly noteworthy that PRIMA-1 can reactivate His-175 mutant p53, which has extensive structural defects[19]. However, one mutant, Phe-176, was not reactivated by PRIMA-1. A probable explanation is that the substitution of Cys-176, one of the four Zn ligands, results in loss of the Zn atom, which is crucial for shaping the DNA-binding domain of p53 (ref. 19). It seems unlikely that this particular defect can be reversed. The observation that PRIMA-1 did not inhibit growth of renal carcinoma KRC/Y cells carrying Phe-176 mutant p53 supports the data from our bandshift assays.

We further confirmed the reactivation of DNA binding by PRIMA-1 in living cells using EGFP and LacZ reporter-based assays with both endogenous and ectopically expressed mutant p53. Moreover, PRIMA-1 induced the endogenous p53 target genes p21 and MDM2 exclusively in the presence of mutant p53 expression. Interestingly, we noticed a reduction in the total levels of p53 protein and a cytoplasmic relocalization of p53 24 h after treatment of cells with PRIMA-1, as shown by immunofluorescence staining and western-blotting (Fig. 3*c* and data not shown). This would be expected if PRIMA-1-mediated reactivation of mutant p53 induces expression of MDM2 that targets p53 for degradation in the cytoplasm. However, the amount of mutant p53 reactivated by PRIMA-1 was apparently sufficient to trigger apoptosis before its degradation by MDM2.

The 22/23 double mutation in the N-terminal transactivation domain abolishes transcriptional transactivation by wild-type p53 (ref. 20). We found that cells expressing His-175 mutant p53 with the same double mutation were substantially less sensitive

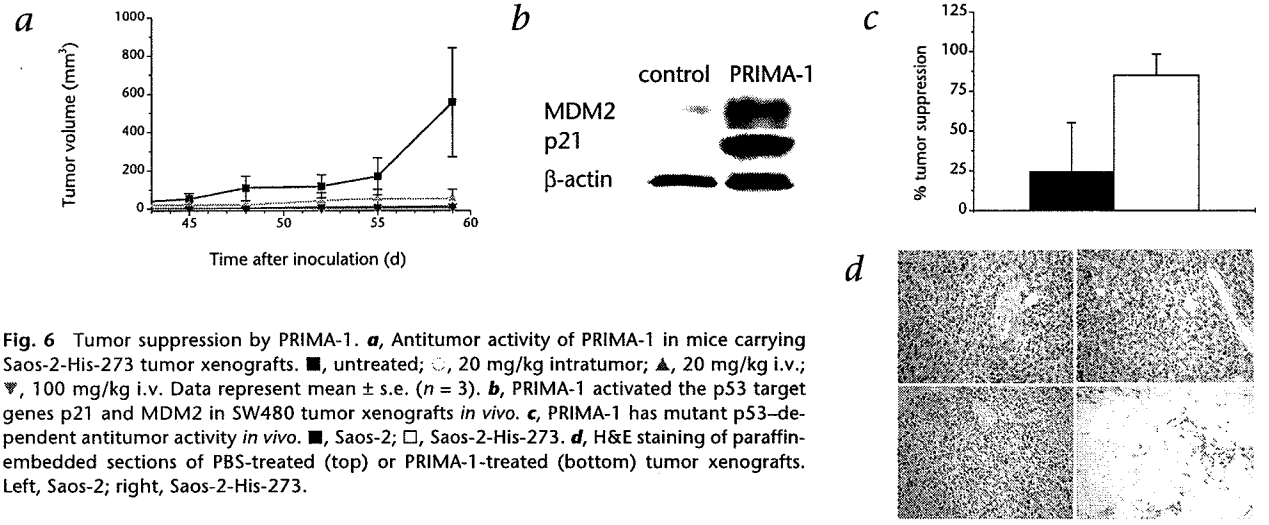


Fig. 6 Tumor suppression by PRIMA-1. ***a***, Antitumor activity of PRIMA-1 in mice carrying Saos-2-His-273 tumor xenografts. ■, untreated; ○, 20 mg/kg intratumor; ▲, 20 mg/kg i.v.; ▼, 100 mg/kg i.v. Data represent mean ± s.e. (n = 3). ***b***, PRIMA-1 activated the p53 target genes p21 and MDM2 in SW480 tumor xenografts *in vivo*. ***c***, PRIMA-1 has mutant p53–dependent antitumor activity *in vivo*. ■, Saos-2; □, Saos-2-His-273. ***d***, H&E staining of paraffin-embedded sections of PBS-treated (top) or PRIMA-1-treated (bottom) tumor xenografts. Left, Saos-2; right, Saos-2-His-273.

to PRIMA-1-induced growth suppression. In addition, pretreatment with cycloheximide abrogated PRIMA-1-induced growth suppression, indicating the requirement for *de novo* protein synthesis. We therefore conclude that restoration of specific DNA binding and transcriptional transactivation function to mutant p53 is critical for induction of apoptosis by PRIMA-1.

The molecular mechanism of mutant p53 reactivation is largely unknown. Structural studies of the mutant p53 core domain predicted that Ser-245 mutant p53 is the most promising with regard to pharmacological rescue whereas globally denatured β-sandwich and Zn-region mutants should be refractory to reactivation[21]. However, experimental data suggest that different types of p53 mutants can be functionally restored[9,22]. It is conceivable that structural analysis of the isolated core domain provides insufficient information because sequences outside the core domain may significantly affect p53 activity[23–28]. This suggests that screening for molecules that only interact with the p53 core domain would be inadequate for the identification of mutant p53–reactivating drugs. Our screening approach should permit the selection of compounds that may have effects on the domains adjacent to the core as well.

Our results demonstrating that PRIMA-1 can restore wild-type conformation to recombinant mutant p53 and preserve the DNA binding of wild-type p53 in the absence of cellular proteins suggest that PRIMA-1 interacts directly with p53. However, it is also possible that PRIMA-1 reactivates p53 by other mechanisms. Further studies—including nuclear magnetic resonance imaging and/or X-ray crystallography analysis—will be required to address this question.

The mechanism behind PRIMA-1-mediated restoration of mutant p53 function, albeit incompletely understood, is fundamentally different from the stabilization of the wild type–specific PAb1620 epitope achieved by CP31398 (ref. 29). Addition of PRIMA-1 to cellular extracts restored the DNA binding to mutant p53 in bandshift assays and PAb1620^{+} conformation in ELISA, suggesting that PRIMA-1 acts on previously unfolded protein. Whereas CP31398 confers protection from thermal denaturation, PRIMA-1 converts the mutant form of the protein into a properly folded active form. Therefore, these two compounds may have entirely different effects in living cells. Whereas PRIMA-1 will force already accumulated mutant p53 to adopt an active conformation, the effect of CP31398 will be restricted to newly synthesized p53.

Comparison of tumor volumes and histological sections of Saos-2 and Saos-2-His-273 xenografts treated with PRIMA-1 demonstrated that the antitumor effect of PRIMA-1 was dependent on mutant p53 expression. Furthermore, PRIMA-1 caused induction of p53 target genes in SW480 xenograft tumors. Taken together, these results support the idea that PRIMA-1 is capable of functional reactivation of mutant p53 *in vivo*. We observed significant tumor suppression following both intratumoral and i.v. administration of PRIMA-1. The effect of i.v. administration suggests that systemic treatment with new drugs based on PRIMA-1 will be feasible. This would be of great importance for treatment of patients with disseminated disease. In order to overcome the potential problem of selection for resistance, it may be necessary to combine PRIMA-1 treatment with other agents to simultaneously attack multiple lesions in tumor cells. Alternatively, PRIMA-1 may act synergistically with conventional chemotherapy or radiotherapy, allowing lower doses of drugs or irradiation for efficient tumor-cell killing.

The identification of a small molecule able to restore biochemical and biological function to mutant p53, resulting in significant tumor suppression *in vivo*, opens exciting prospects for future cancer therapy. A more detailed investigation of the molecular mechanism behind PRIMA-1-mediated reactivation of mutant p53 may provide a basis for the design of new potent and tumor-specific drugs.

Methods

Cells and plasmids. The human Saos-2-His-273 osteosarcoma, H1299-His-175 lung adenocarcinoma, and SKOV-His-175, His-273 and His-175-22/23 ovarian carcinoma-cell lines carry the indicated tetracycline-regulated mutant p53 constructs. The His-175-22/23 mutant p53 has substitution of Leu 22/Trp 23 for Gln/Ser in the transactivation domain. The human HCT-116 colon carcinoma-cell line carries wild-type p53 and the A431 colon carcinoma-cell line carries His-273 mutant p53 and a p53-responsive LacZ reporter. Other cell lines used are indicated in Supplementary Table A. The plasmids encoding the GST-p53 fusion proteins have been described[31]. The p53-EGFP plasmid contains 13 synthetic p53 consensus DNA binding sites in front of the EGFP coding sequence. Transient transfection experiments were performed with Lipofectamine 2000 according to the manufacturer's recommendations (Invitrogen Life Technologies, Groningen, the Netherlands).

Screening of a chemical library and growth suppression assays. A library of low-molecular-weight compounds was obtained from the National Cancer Institute (NCI), Bethesda, Maryland. For more information, see http://dtp.nci.nih.gov. Saos-2-His-273 cells grown in the presence or absence of doxycycline were treated with compounds from the library (Diversity Set) at a concentration of 25 μM in 96-well plates. Growth suppression was assessed by the WST-1-cell proliferation reagent (Roche Diagnostics, Bromma, Sweden) after 48 h incubation. Absorbance of samples was measured at 490 nm.

***In vitro* assays.** For FACS analysis, cells were stained with propidium iodide and analyzed on a Becton Dickinson FACScan (Mountain View, California) according to standard procedures. TUNEL staining, immunostaining, LacZ staining, preparation of cell extracts, ELISA and western-blotting were performed according to standard procedures. GST–p53 proteins were prepared and analyzed for specific DNA binding as described[13,31]. For PAb1620 staining, cells were fixed with 4% formaldehyde. The anti-p53 monoclonal antibodies PAb1620, PAb240 and PAb1801 were obtained from Calbiochem (Darmstadt, Germany). The anti-p53 rabbit polyclonal antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, California), the anti-MDM2 monoclonal antibody was from Neo Markers (Fremont, California) and the anti-p21 monoclonal antibody was from Transduction Laboratories (Lexington, Kentucky). Secondary antibodies (FITC-conjugated horse anti-mouse immunoglobulin, Texas Red-conjugated goat anti-rabbit immunoglobulin) were from Vector (Burlingame, California). All other reagents were from Sigma–Aldrich Sweden AB (Stockholm, Sweden).

Animal studies. All animal studies were approved by the local animal ethical committee in Stockholm, Sweden, and animal care was in accordance with institutional guidelines. For toxicity assessment, 12 SCID mice (average weight, 20 g) were divided into 4 groups. 3 groups received daily i.v. injections of 1, 10 and 100 mg/kg of PRIMA-1 in PBS for 5 d. Control animals were injected with PBS. Changes in body weight were monitored for 1 mo after the last injection. For assessment of the antitumor activity of PRIMA-1, 12 SCID mice were inoculated with 1×10^6 Saos-2-His-273 cells in 90% Matrigel (Becton Dickinson, Le Pont-De-Claix, France) subcutaneously and unilaterally into the right flanks. After 3 d, mice were divided into 4 groups. 2 groups received i.v. injections of PRIMA-1 at a dose of either 20 or 100 mg/kg, 1 group received intratumor injections of PRIMA-1 at a dose of 20 mg/kg, and the last group was used as a control. Injections were performed twice daily for 3 d. Tumor volume was measured at 2 mo.

PRIMA-1 mediated activation of p53 target genes *in vivo* was assessed by western-blot analysis using extracts from SW480 xenografts excised from mice 16 h after intratumor injection. To compare the effect of PRIMA-1 on p53-null and mutant p53-expressing xenograft tumors, 10 SCID mice were inoculated with 1×10^6 Saos-2 and Saos-2-His-273 in 90% Matrigel in the right and left flanks, respectively. After 5 d, mice were treated twice a day with i.v. injections of PRIMA-1 (100 mg/kg) or PBS for 10 d. Paraffin-embedded tumor sections were stained with H&E. In all experiments, mean tumor volumes in treated and control animals were compared at a given time point using the Wilcoxon matched-pairs test.

Note: Supplementary information is available on the Nature Medicine *website (http://medicine.nature.com/supplementary_info/).*

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Competing interests statement

The authors declare that they have no competing financial interests.

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APPENDIX D

[CANCER RESEARCH 50, 6757-6764, October 15, 1990]

Integrin Distribution in Malignant Melanoma: Association of the β_3 Subunit with Tumor Progression[1]

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ABSTRACT

Since tumor progression is dependent on the ability of malignant cells to interact with the extracellular matrix, molecules on the cell surface which mediate cell-substratum interactions are likely to be important regulators of tumor invasion and metastasis. The purpose of this study was to examine the distribution of one such group of cell adhesion receptors, the integrins, in benign and malignant lesions of human melanocytes. The distribution of integrin adhesion receptors was defined on cells in culture derived from normal and malignant melanocytes and in tissue sections from benign to increasingly malignant melanocytic lesions using a panel of monoclonal antibodies against specific integrin subunits. Cells in culture expressed a large variety of integrins, including all of the previously characterized members of the β_1 subfamily plus the α_v/β_3 vitronectin receptor. The expression of integrins was similar in cells cultured from either benign or malignant lesions. In contrast, consistent differences were noted in integrin expression by cells within tissues containing metastatic and vertical growth phase melanomas when compared to radial growth phase melanoma cells and cells within nevi. Most notably, the expression of the β_3 subunit was restricted exclusively to cells within vertical growth phase and metastatic melanomas. The presence of this integrin may be important in the development of tumor invasiveness and could be useful as a marker of melanoma cells entering the more aggressive phase of the malignant process.

INTRODUCTION

The process of tumor invasion and metastasis requires complex changes in normal cell-cell and cell-substratum interactions (1, 2). In order for tumor cells to migrate through adjacent tissues and become invasive, normal cell contacts must be broken and the cells must be able to attach efficiently to the extracellular matrix proteins of the surrounding stroma (1). The cellular receptors mediating these adhesive events are thus likely to be important in tumor invasion and metastasis (2, 3).

One family of cell surface proteins that participates in cell adhesion and migration is the integrins (4–7). Structurally, each integrin is a heterodimer consisting of an α subunit noncovalently associated with a β subunit. The receptor complex spans the plasma membrane, linking the internal cytoskeletal network of a cell with the external extracellular matrix (4). Specificity for ligand binding is determined by the particular combination of α and β subunits. The integrins are divided into at least 5 subfamilies, each being defined by a common β subunit (5). The best characterized subfamilies are the β_1 subfamily, which includes receptors for laminin, fibronectin, and collagen; the β_2 subfamily, found on leukocytes, which includes receptors mediating cell-cell interactions; and the β_3 subfamily, which includes the platelet glycoprotein IIb/IIIa complex and the "vitronectin" receptor which also binds fibrinogen, thrombospondin, and von Willebrand's factor (8, 9).

Data from *in vitro* and *in vivo* comparisons of control and malignant cells have suggested that changes in integrin expression accompany malignant transformation. However, no uniform pattern of change has emerged. Rodent cells transformed with Rous sarcoma virus (10), as well as basal cell or squamous cell carcinomas (11), show a reduction in the expression of integrins from the β_1 subfamily. In contrast, chemically transformed, tumorigenic human osteosarcoma cells display an increase in β_1 integrins when compared to nontumorigenic osteosarcoma cells (12). Other transformed cells show an alteration in the distribution of integrins with no sign of changes in their expression (13).

Despite the lack of consistent changes in the pattern of integrin expression that correlate with tumorigenesis, functional studies suggest that integrins are important in the metastatic process. Synthetic peptides containing the amino acid sequence RGD[3] (derived from one of the cell-binding domains of fibronectin and other matrix proteins) that block the binding of many integrins to their extracellular matrix ligands (14, 15) inhibit the movement of human melanoma cells through an amniotic basement membrane in an experimental model of invasion (16). These peptides also reduce the number of metastatic nodules found within the lungs of mice given B16F10 melanoma cells (17–19). Consistent with these studies is the observation that antibodies against the β_3 subfamily of integrins prevent the establishment of tumors when human melanoma cells are implanted into nude mice (20). Finally, the overexpression of the fibronectin receptor, $\alpha_5\beta_1$, reduces the ability of Chinese hamster ovary cells to form tumors in nude mice (21).

The purpose of this study was to examine the distribution of integrins in a well-characterized system of human tumor progression which results in the development of malignant melanoma (22, 23). A panel of antibodies directed against specific integrin subunits was used to characterize the integrin repertoire of cells from human melanocytic neoplasms in cell culture and in tissue sections. Since cultured cells explanted from all stages of tumor development have been established and characterized (24–27), this approach provided the opportunity to compare integrin expression of malignant cells in culture with those *in situ*, as well as to monitor integrin expression during tumor progression.

MATERIALS AND METHODS

Antibodies. Both Mabs and polyclonal antibodies directed against various subunits of different integrin receptors were used for immuno-

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[3] The abbreviations used are: RGD, Arg-Gly-Asp; Mab, monoclonal antibody; SDS, sodium dodecyl sulfate; RGP, radial growth phase; VGP, vertical growth phase; AEC, aminoethylcarbazole.

precipitation. Only Mabs were used for tissue staining. Mabs P1H5, P1B5 and P1D6 (recognizing α_2, α_3, and α_5 subunits, respectively) were generously provided by Drs. Elizabeth Wayner and William Carter (28, 29). Dr. Martin Hemler kindly supplied the Mabs TS2/7 and B-5H10 directed against the α_1 and α_4 subunits, respectively (30, 31). Dr. Arnoud Sonnenberg donated the GoH3 monoclonal antibody directed against the α_6 integrin (32). Drs. Joel Bennett and James Hoxie provided the SSA6 Mab that is directed against the β_3 subunit and B1B5 directed against platelet glycoprotein IIb (α_{IIb})(33). The Mab LM142, directed against the vitronectin receptor α subunit (α_v), was donated by Dr. David Cheresh (34). The polyclonal antibody raised against the 140-kDa adhesion receptor complex in rat L6A cells which reacts against integrins in the $\beta 1$ and β_3 subfamilies has been previously described (35).

Cell Lines. Sixteen different cell lines derived from primary and metastatic melanomas were examined. In addition, four lines of melanocytes explanted from normal skin and congenital nevi were studied. Cultured melanocytes were isolated from foreskins of newborn humans as previously described (26). The human nevus and tumor cells used in these studies have been characterized elsewhere (24–27). Cells were routinely cultured in MCDB153/L-15 medium supplemented with fetal calf serum, pituitary extract, insulin, and transferrin (26). In five cases, melanoma cell lines derived from both the primary melanoma and a metastatic lesion from the same patient were available.

Labeling of Cells. For ^{125}I labeling, intact monolayers of cells in 25-cm^2 tissue culture flasks were washed with phosphate-buffered saline and exposed sequentially to 100 units/ml of lactoperoxidase (Sigma Chemical Co., St. Louis, MO), 1 mCi of carrier-free ^{125}I (Amersham, Arlington Heights, IL), and three 40-μl aliquots of 0.06% hydrogen peroxide. The cells were harvested and extracted as described below.

Cell Harvest and Nonidet P-40 Extractions. After being washed three times with phosphate-buffered saline, labeled cells were extracted by exposing the monolayers to 0.5–1.0 ml of 0.01 M Tris acetate buffer, pH 8.0, containing 0.5% Nonidet P-40, 0.5 mM Ca^{2+}, and phenylmethylsulfonyl fluoride at a 2 mM concentration. Cells were then scraped from the vessel and the extraction continued for 20 min at 4°C. The extract was centrifuged for 30 min at 12,000 × g and the resulting supernatant used for immunoprecipitation.

Immunoprecipitation and Gel Electrophoresis. Nonionic detergent extracts were preadsorbed for 30 min at 4°C with protein G conjugated to Sepharose beads (Pharmacia, Piscataway, NJ). Fifty μl of the appropriate antibody was then added to 100 μl of the extract and the mixture allowed to stand for 1 h at 4°C. Immunocomplexes were collected by adsorption onto protein G Sepharose beads for 1 h at 4°C. The beads containing the complex were washed 5 times with a buffer containing 50 mM Tris HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 5% deoxycholate, and 0.1% SDS. The beads were then suspended in an electrophoresis sample buffer [62.5 mM Tris base, 2% SDS, 10% glycerol (pH 6.8)]. The material eluted from the beads was analyzed by SDS-polyacrylamide gel electrophoresis using 6% polyacrylamide gels as previously described (35) under nonreducing conditions. Gels were dried and exposed to Kodak XR-5 X-ray film at −70°C.

Origin and Classification of Tissue Samples. All tissue samples were obtained from diagnostic or therapeutic biopsies taken from patients in the Pigmented Lesion Group, Hospital of the University of Pennsylvania. The samples were snap frozen in liquid nitrogen and stored at −70°C until the time of sectioning. The biopsy specimens were classified by standard histological criteria (22, 23, 36) as belonging to one of the following groups: benign nevus, primary melanoma (either radial or vertical growth phase), or metastatic melanoma. Nevus cells seen in the benign melanocytic nevus were found as collections of cuboidal cells either in the dermis or at the dermal-epidermal junction without any suggestion of nuclear atypia or inflammatory response. Primary melanomas were divided into RGP or VGP (22, 23, 36). RGP melanomas were defined as proliferations in the epidermis of moderately to severely atypical melanocytic cells with or without small clusters of lesional cells in the dermis without proliferation. The VGP primary melanomas were invasive into the dermis in proliferative clusters or in sheets and tended to show greater atypia than RGP cells. The VGP melanomas formed clinically and histologically recognizable tumor masses at the primary site and may therefore be considered "tumorigenic" primary melanomas. Metastatic melanomas resembled the VGP cells both histologically and cytologically, but these tumorigenic lesions were found at sites distant from the primary lesion. Some specimens revealed both the RGP and VGP compartments. In these cases, each portion of the lesion was analyzed separately.

Immunohistochemistry. Cryostat sections 4- to 8-μm thick were placed on poly-L-lysine-coated slides and fixed in −20°C acetone for 10 min. The peroxidase-based Vectastain Elite ABC system (Vector Laboratories, Burlingame, CA) was used to detect the monoclonal anti-integrin antibodies. In lesions with large amounts of brown melanin pigment, the red AEC substrate was used for contrast. These sections were lightly counterstained with Mayer's hematoxylin. For the less pigmented nevus cells, diaminobenzidine was used as the the peroxidase enzyme substrate without the use of counterstaining. Each lesion was classified using the criteria described above and the degree of immunoperoxidase staining of the melanocytes or melanoma cells was graded negative, weak staining, or strong staining. Staining was considered positive for an entire lesion if 10% or more of the cells showed strong staining. For reference purposes, the staining shown by the nevus cells in Fig. 2, *F* and *G*, were graded as strongly positive, the nevus cells in Fig. 2, *C* and *D*, as weakly positive, and the nevus cells in Fig. 2, *H* and *I*, as negative. The percentage of cells positive with any of the monoclonal antibodies was visually estimated. Color slides were taken using Ektachrome 64 indoor film. For black and white pictures, Kodak T-MAX 400 film was used.

Statistical Analysis. The proportion of tumorigenic *versus* nontumorigenic lesions expressing β_3 and α_4 integrin subunits were analyzed using χ^2 analysis.

RESULTS

Integrin Expression by Cultured Melanocytes and Melanoma Cells. To determine the integrin profile of melanocytes and melanoma cells in culture, extracts of ^{125}I-labeled cell of four lines of normal melanocytes (established from fetal foreskin and benign nevi) and 16 different tumor lines (isolated from primary or metastatic melanomas) were subjected to immunoprecipitation. Examples of immunoprecipitations from one nonmalignant melanocyte line (nevus cell line 1692) and one malignant melanoma cell line (line 164) are shown in Fig. 1. Two radioactive bands are seen in the immunoprecipitations using monoclonal antibodies since antibodies specific for one integrin subunit coprecipitate the associated subunit. Thus, monoclonal antibodies against specific integrin α subunits coprecipitated the common 120-kDa β_1 subunit. Similarly, an anti-α_v subunit monoclonal antibody coimmunoprecipitated the 95-kDa β_3 subunit (data not shown) and the anti-β_3 monoclonal antibody coprecipitated the 150-kDa α_v subunit. In the case of nevus 1692 (Fig. 1, *top*), the major integrins detected were $\alpha_1\beta_1$, a collagen/laminin receptor; $\alpha_3\beta_1$, a promiscuous receptor for laminin, fibronectin, and collagen; $\alpha_5\beta_1$, a fibronectin receptor; and $\alpha_v\beta_3$, a vitronectin receptor. No reactivity with an antibody directed against the platelet glycoprotein IIb was detected. Cultured cells derived from the metastatic melanoma 164 expressed more integrins (Fig. 1, *bottom*) than did the nevus cell line 1692, including all the integrins found on the melanocytes plus the $\alpha_2\beta_1$ collagen/laminin receptor and $\alpha_4\beta_1$, a receptor shown to bind to a non-RGD containing region of fibronectin (37) and function in cell-cell interactions (38).

These comparisons suggested that differences between melanocytes and melanoma cells in culture might exist. However, when the immunoprecipitation data from all 20 cell lines were examined (Table 1), no consistent differences between benign and malignant cells types could be detected. There was clearly

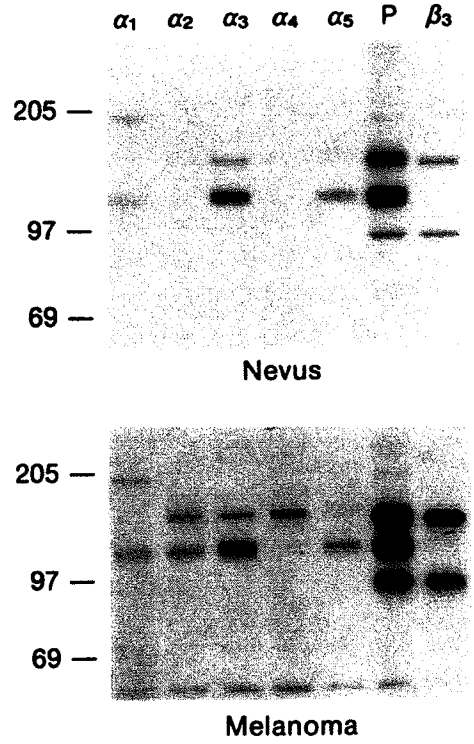

Fig. 1. Immunoprecipitation of integrin subunits from cultured melanocytes and malignant melanoma cells. Nonionic detergent extracts from ^{125}I-labeled melanocytes derived from a nevus (*top*) or a metastatic melanoma (*bottom*) were immunoprecipitated as described in the "Materials and Methods" using monoclonal antibodies directed against five integrin α subunits or the β_3 subunit. Lane *P* (Pan-integrin), immunoprecipitation with a polyclonal antibody which reacts with all integrins from the β_1 and β_3 subfamilies. Each lane is designated according to the integrin α or β subunit that reacted with the monoclonal antibody used in the immunoprecipitation. *Ordinate*, positions and size of molecular mass markers in kDa.

Table 1 *Integrin expression in cell lines*

The distribution of integrin expression in cell lines derived from various types of melanocytic tissues was determined by immunoprecipitation of extracts from ^{125}I-labeled cells using a panel of antibodies. The amount of integrin expression was graded as being present in large amounts (●), being present in trace amounts (*), or being nondetectable (○).

	Integrin subunit						
Tissue	α_1	α_2	α_3	α_4	α_5	β_3	Anti-VNR[a]
Melanocyte							
FM 713	*	○	●	○	*	●	●
FM 624	*	*	●	ND	○	●	●
Nevus							
1692[b]	●	○	●	○	●	●	●
1559	*	*	●	ND	*	●	●
Melanoma							
278 (P)[c]	●	*	●	●	*	●	●
1617 (M)	●	●	●	●	●	●	●
983A (P)[c]	●	*	●	●	*	●	●
983B (M)	●	*	●	*	*	●	●
115 (P)[c]	○	*	●	●	○	●	●
239A (M)	●	*	●	●	*	●	●
1361A (P)[c]	○	*	●	*	*	●	●
1361C (M)	●	●	●	●	*	●	●
75 (P)[c]	●	*	●	*	*	●	●
373 (M)	●	*	●	○	*	●	●
902B (P)	●	○	●	*	○	●	●
793 (P)	*	○	●	●	○	●	●
164[d] (M)	●	●	●	●	●	●	●
451 (M)	○	*	●	*	○	●	●
852 (M)	●	●	●	*	*	●	●
853-2 (M)	●	*	●	●	○	●	●

[a] Anti-VNR, polyclonal antibody against the vitronectin receptor; ND, not determined; P, cells explanted from primary malignant melanomas; M, cells explanted from metastatic malignant melanomas.
[b] Nevus cell line pictured in Fig. 1.
[c] Cell lines explanted from a primary and malignant lesion obtained from the same patient.
[d] Melanoma cell line pictured in Fig. 1.

a high degree of heterogeneity in integrin expression among cell lines. The principal integrins expressed by all the cultured cells were $\alpha_3\beta_1$ and $\alpha_v\beta_3$. The $\alpha_1\beta_1$ collagen/laminin receptor was expressed by many of the melanoma cells and by one of the nevus cell lines at relatively high levels. There was considerable variation in the expression of other integrins. Most cells expressed only trace levels of the classical fibronectin receptor, $\alpha_5\beta_1$, or the collagen/laminin receptor, $\alpha_2\beta_1$. The frequent expression of $\alpha_4\beta_1$ by melanoma cells is particularly interesting, since this integrin has been shown to be involved in cell-cell interactions and could thus possibly play a role in endothelial cell adhesion and extravasation of metastatic cells.

Expression of Integrins on Normal and Malignant Cells in Tissue Sections. The fact that no consistent differences between tumorigenic and nontumorigenic cells in culture were noted did not rule out the possibility that such a difference existed in tissue. Therefore, the integrin profiles of melanocytes and melanoma cells in tissue were compared by immunoperoxidase staining of cryostat sections from 42 different lesions representative of different stages of melanoma progression including benign nevi, RGP primary, VGP primary, and metastatic melanoma (see "Materials and Methods" for details of tumor classification).

The integrins expressed by cells within each cryostat section were determined using antibodies specific for the α subunit of 6 receptors in the β_1 integrin subfamily, the α subunit of the vitronectin receptor (α_v), and the β_3 subunit. An example of the staining pattern of skin containing a benign congenital nevus is illustrated in Fig. 2. The staining in the epidermis (*arrows*) was primarily confined to the basal layers. The keratinocytes stained strongly for the α_2 (Fig. 2*B*), α_3 (Fig. 2*C*), and α_6 (Fig. 2*F*) subunits. There was some expression of α_1 (Fig. 2*A*) and α_v (Fig. 2*G*), but staining with antibodies against α_4 (Fig. 2*D*), α_5 (Fig. 2*E*), and β_3 (Fig. 2*H*) did not exceed the background noted in control sections stained with an antibody against an irrelevant antigen (Fig. 2*I*).

The staining pattern of a benign melanocytic nevus (an example of one of six benign melanocytic nevi studied in detail) is also seen in the same sections (Fig. 2). The nevus cells (*arrowheads*) strongly expressed α_6 (Fig. 2*F*) and α_v (Fig. 2*G*). The expression of α_1 (Fig. 2*A*) was less intense and roughly equivalent to that of the basal keratinocytes in the same section. Some expression of α_2, α_3, and α_4 was noted (Fig. 2, *B, C,* and *D*), but staining was less intensive and patchy in its distribution. On average, 40% of the cells within a given nevus ($n = 6$) were graded as positive for α_2 and 28% for α_3. The nevus illustrated in Fig. 2 was the only one of six to show α_4 staining, with approximately 30% of the nevus cells in the deeper regions of the dermis being positive. Staining of sections from all six nevi for α_5 and β_3 (Fig. 2, *E* and *H*) did not exceed that of the nonspecific background noted in the control sections (Fig. 2*I*).

In general, the expression of the β_1 integrins by RGP ($n = 6$), VGP ($n = 5$), and metastatic cells ($n = 5$) was qualitatively similar to that of nevus cells (data not shown). The α_1 and α_6 subunits were consistently present on all of the malignant cells. The expression of the α_2 and α_3 subunits remained somewhat

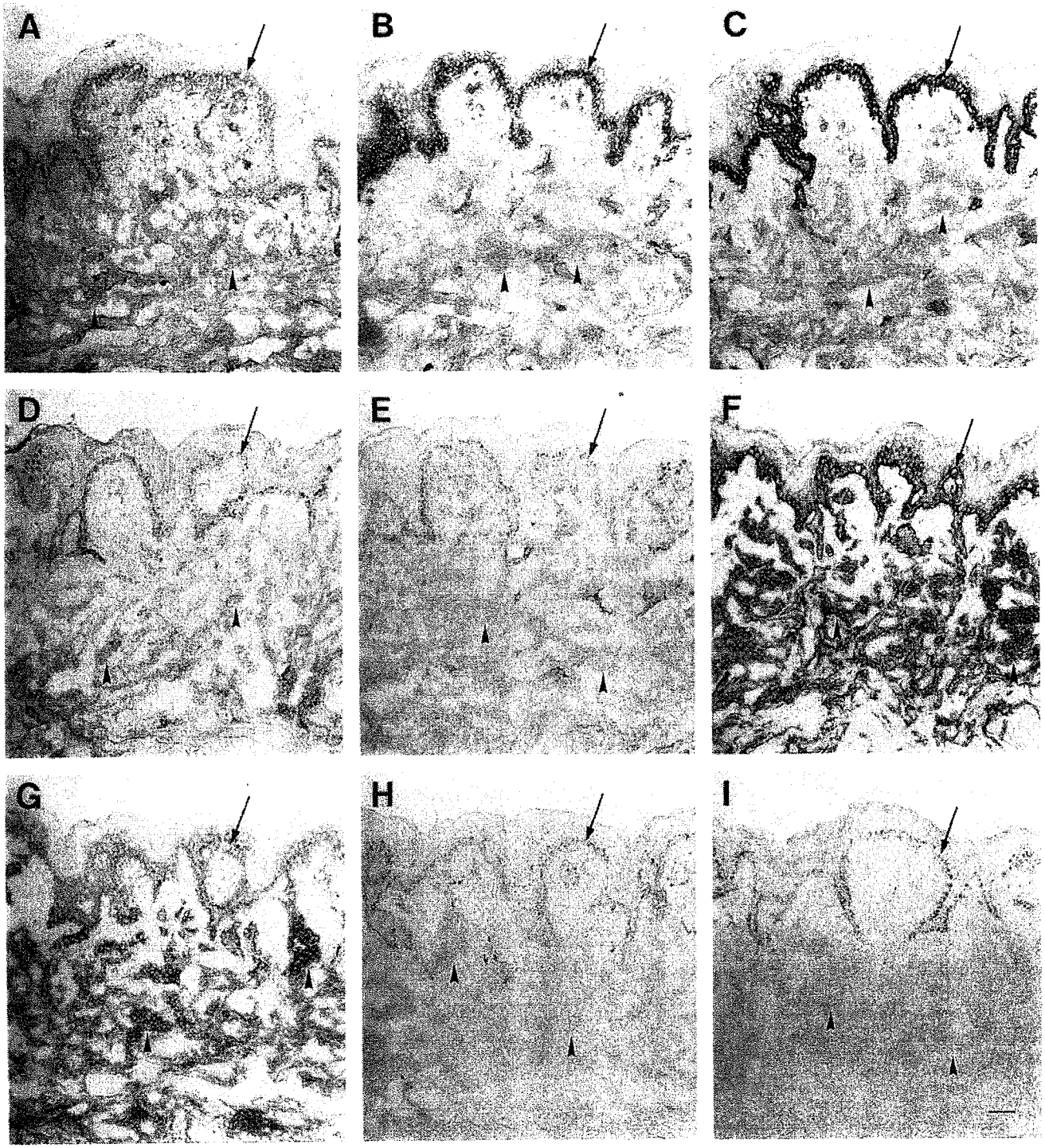

Fig. 2. Tissue distribution of integrins in normal skin and a congenital nevus. Frozen sections of a portion of skin containing a congenital nevus were exposed to monoclonal antibodies directed against one of six α subunits in the β_1 subfamily of integrins, the α_v and β_3 subunits, and a control antibody. The sections were then treated with biotinylated anti-mouse IgG, exposed to avidin-peroxidase complexes, and reacted with diaminobenzadine as the chromagen to identify cells reacting with the monoclonal antibodies. No counterstaining was used. The staining pattern of the basal layer of keratinocytes in the epidermis (*arrows*) can be contrasted with that of the nevus cells which are infiltrating the underlying dermis (*arrowheads*). *Bar*, 100 μm. *A*, anti-α_1; *B*, anti-α_2; *C*, anti-α_3; *D*, anti-α_4; *E*, anti-α_5; *F*, anti-α_6; *G*, anti-α_v; *H*, anti-β_3; *I*, control. Dark areas at the basal layer of the epidermis in sections *D*, *E*, *H*, and *I* (*arrows*) represent melanin deposition in contrast to the diffuse cellular staining seen in sections *A*, *B*, *C*, *F*, and *G*.

heterogeneous, but the percentage of cells within a given lesion that were positive for the α_2 (78% of cells) and α_3 (73% of cells) subunits was higher than in the nevus tissues. The α_5 fibronectin receptor was found in only three samples (two VGP lesions and one metastasis). There appeared to be a difference between the nontumorigenic (nevus and RGP melanomas) and tumorigenic (VGP and metastatic melanomas) lesions with regard to the expression of the α_4 subunit. On our initial survey, the α_4 subunit was expressed in a focal distribution on approximately half of the VGP and metastatic tumors but on only one RGP

melanoma and one of the nevi. Because of this difference, we screened an additional 6 nevi, 7 VGP lesions, and 6 metastatic tumors for the presence of the α_4 subunit. As shown in Table 2, only 2 of 18 (11%) of the nontumorigenic lesions expressed the α_4 subunit as compared with 9 of 23 (39%) tumorigenic neoplasms. This difference was significant at the $P < 0.05$ level.

The most striking difference noted between tumorigenic and nontumorigenic lesions was the marked contrast in expression of the β_3 subunit. Whereas the cells from nevi, RGP, VGP, and metastatic lesions all stained strongly with anti-α_v antibody, only tumorigenic VGP and metastatic melanoma cells expressed the β_3 subunit. The benign melanocytic nevus and nontumorigenic RGP primary melanoma uniformly failed to express this subunit. Sections selected from each of these pathological classifications, stained with a monoclonal antibody (SSA6) specific for the β_3 subunit are shown in Fig. 3. The loosely packed cluster of cells within the nevus failed to react with this antibody (Fig. 3*A*). Likewise, the cluster of melanoma cells within the nontumorigenic RGP primary melanoma did not react with this antibody (Fig. 3*B*). In marked contrast, VGP cells and metastatic cells stained strongly with this antibody (Fig. 3, *C* and *D*). No staining was observed using an antibody against the α_{IIb} subunit which is found in association with β_3 in platelets (data not shown).

Table 2 *Expression of integrin subunits in melanocytic tissue*

Tissue	Integrin subunit	
	α_4	β_3
Nevus	1/6 (30)[a]	0/9
RGP primary melanoma	1/12 (80)	0/12
VGP primary melanoma	4/12 (55 ± 8)	8/10 (61 ± 9)
Metastatic melanoma	5/11 (78 ± 6)	11/11 (66 ± 15)

[a] Numerator, number of lesions where at least 10% of the cells demonstrated strong staining by immunohistocytochemistry; denominator, number of lesions examined; numbers in parentheses, percentage of cells (±SD) within each lesion that reacted with a given antibody.

Fig. 4*A* is an overview of a complex primary melanoma that contained regions of invasive VGP cells (*left*), as well as regions of RGP cells (*right*), confined to the epidermis and papillary dermis. The positive reaction with the anti-β_3 monoclonal antibody was evidenced in the areas showing red and was limited to cells within the VGP portion of the tumor. The red of the positive AEC-staining cells could easily be distinguished from the brown of the melanin-containing tumor cells. Although many of these VGP cells expressed the β_3 integrin receptor, heterogeneity of staining was apparent. At lower magnification (Fig. 4*A*) certain regions within the VGP portion of the lesion were clearly reactive with antibodies against β_3 integrins, while other regions showed more heterogeneity in their staining pattern. When examined at higher magnification (Fig. 4*B*), heterogeneity was still apparent, but positively staining cells could be readily detected. This heterogeneity was also seen in other lesions examined. Between 30 and 90% of the cells within a single VGP or metastatic lesion reacted with monoclonal antibodies specific for β_3 integrins. All cells within the RGP portions of this same lesion (Fig. 4*C*) consistently failed to show any reactivity with the anti-β_3 Mab. The brown is due to the melanin produced by these cells.

Because of the striking differences in β_3 expression between nontumorigenic and tumorigenic melanocytes, we expanded our initial survey to include 42 lesions. These results are sum-

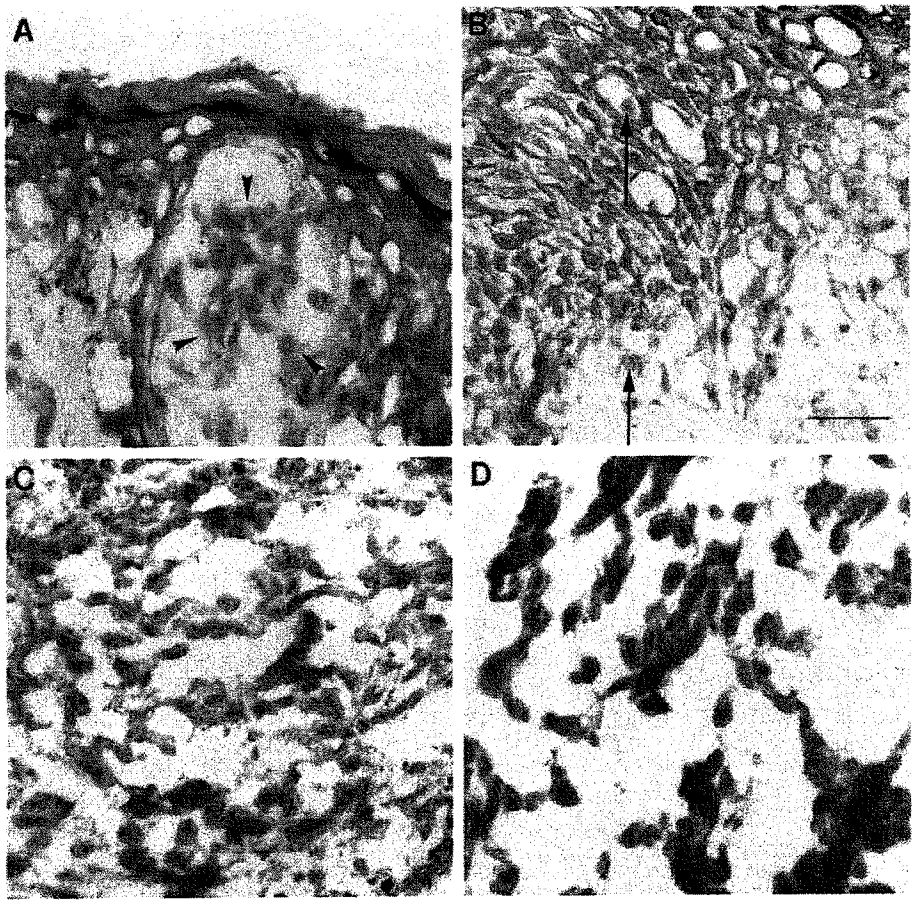

Fig. 3. Tissue distribution of β_3 integrins in melanotic lesions. Frozen sections were stained with an anti-β_3 integrin subunit monoclonal antibody and counterstained by the immunoperoxidase technique using the red AEC chromagen to distinguish reactivity from the brown of melanin (see "Materials and Methods"). *Bar*, 100 μm. *A*, congenital nevus (*arrowheads*); *B*, radial growth phase of a lentigo maligna melanoma containing keratinocytes and and a few neoplastic melanocytes (*arrows*); *C*, vertical growth phase primary melanoma; *D*, metastatic melanoma. There is no reactivity against nevus cells, radial growth phase melanoma cells, or keratinocytes (*A* and *B*). In contrast, vertical growth phase cells and metastatic melanoma cells show evidence of red indicating reactivity with the anti-β_3 monoclonal antibody (*C* and *D*).

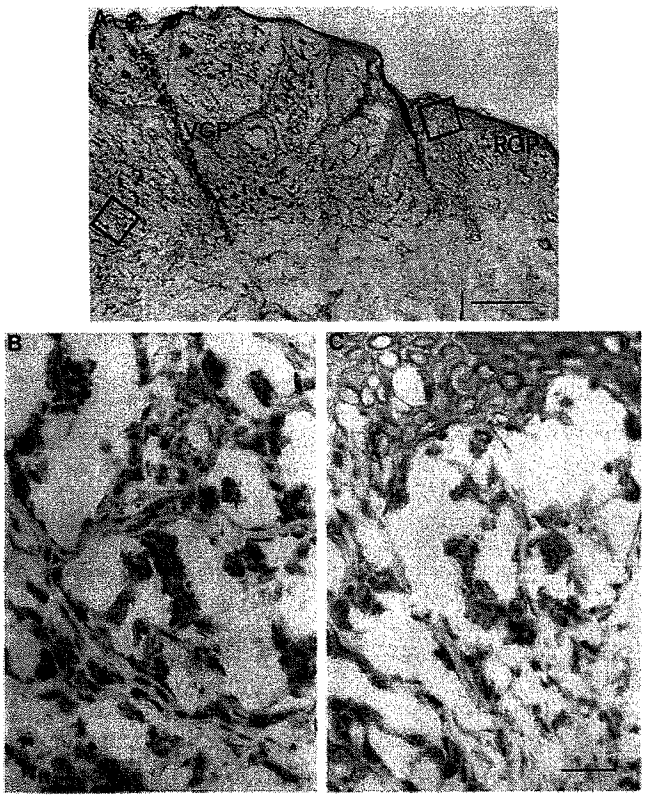

Fig. 4. Distribution of β_3 integrins within a complex primary melanoma. Frozen sections of a complex primary melanoma were stained with the anti-β_3 monoclonal antibody and counterstained by the immunoperoxidase technique using the red AEC chromagen (see "Materials and Methods") to contrast with the brown melanin pigment. In *A*, lesion presents with a VGP nodule that elevates the epidermis (*left*). The nodule is contiguous with a flat RGP (*right*); *bars*, 500 μm. *Insets*, approximate location of fields depicted in Fig. 3, *B* and *C*; *bar*, 100 μm. In *B*, VGP cells contain both brown melanin and red AEC chromagen, indicating reactivity with the anti-β_3 monoclonal antibody. In *C*, RGP cells contain only brown melanin pigment and no red, indicating negative reactivity with the anti-β_3 Mab.

marized in Table 2. Zero of 9 nevi and 0 of 12 RGP lesions expressed the β_3 subunit. In contrast, 8 of 10 VGP lesions (80%) and 11 of 11 (100%) metastatic melanomas stained positively with anti-β_3 antibody. The difference between tumorigenic *versus* nontumorigenic β_3 integrin expression was highly significant ($P < 0.001$).

DISCUSSION

The interaction of tumor cells with extracellular matrix has long been suspected as an important component of tumorigenicity and metastasis (reviewed in Refs. 1 and 3). A comparative analysis of specific cell-matrix adhesion receptors, such as the integrins, present on tumor cells and nontransformed cells may thus be important to our understanding of tumor progression. A number of investigators have begun to study the types and functions of integrins on a variety of tumor cells in culture and have found various changes in integrin expression or function (see "Introduction"). However, to date, no studies have comprehensively examined the distribution of integrins in a well-defined system of tumor progression or compared *in vitro* to *in situ* integrin expression. Because the development of melanomas takes place in well-defined steps (22, 23), and these cells are easily grown in culture, this system is ideally suited for meaningful comparative studies of integrin expression.

Melanocytes and melanoma cells in culture expressed a wide variety of integrins. These included all of the known β_1 or VLA subfamily of integrins plus α_v/β_3. Examination of Table 1 highlights the heterogeneity of integrin expression observed among various cell lines. Although all cells produced readily detectable amounts of the the α_3, α_v, and β_3 subunits, the expression of other integrins was quite variable. Because of this heterogeneity, it was difficult to identify clear differences between the integrin repertoire of melanocytes derived from normal skin and nevi compared with those derived from primary or metastatic melanomas, with the possible exception of an increased expression of the α_4 subunit on cells derived from malignant tumors. Although the significance of this difference is unclear due to the limited number of nonmalignant cell lines studied, the presence of the α_4/β_1 integrin on melanoma cells in culture and in tissues is especially interesting, since this receptor (also known as VLA-4) has been implicated in cell-cell adhesion. The ligand for VLA-4 is an endothelial cell surface protein, VCAM-1 (vascular cell adhesion molecule-1) (37, 38), that appears to be similar or identical to INCAM-110, a recently described inducible endothelial cell surface glycoprotein that mediates the adhesion of certain human melanoma cell lines to cultured endothelium (39). The presence of $\alpha_4\beta_1$ on some of the cultured human melanoma cell lines studied here and on about one-third of the malignant melanoma cells in tissues supports the idea that it may play a role in the metastatic process, although the absence of this receptor on many cell lines and tumors indicates that it is not necessary for metastasis.

Some of the integrins reported here have also been noted by others studying individual melanoma cell lines, both human and murine in origin. Among the integrins expressed in these lines were α_1/β_1 and α_2/β_1 (40), α_4/β_1 (41), and α_6/β_1 (42), as well as the β_3 and α_v subunits (8). These results, taken with those reported here, support the diverse pattern of integrins expressed in culture.

Since the expression of many cell surface proteins is markedly altered by the process of tissue culture (26), it was important to establish the relationship between the distribution of of integrins on cells in culture *versus* those that reside in tissue. The most important finding of this study was the contrast in expression of the β_3 integrins by benign melanocytes and melanomas in the radial growth phase (nontumorigenic lesions) *versus* those melanoma cells in tumorigenic lesions, *i.e.*, the vertical growth phase of primary melanoma or metastatic tumors. Cells within almost all of the VGP and metastatic lesions expressed the β_3 integrin, while benign melanocytes and cells within RGP melanomas did not. In every positive case, between 30 and 90% of cells within a given lesion stained strongly for the β_3 subunit. This pattern of heterogenous expression was in contrast to the more uniform expression of the β_1 subfamily of integrins by epidermal cells but was similar to the the patchy distribution of the α_2 and α_3 subunits by melanomas and melanocytes. Although the reason for the heterogeneity of β_3 receptor expression is not known, heterogenous expression of tumor antigens has been well described in malignant melanoma (43) and many other tumors (44, 45).

These findings agree with and extend those of McGregor *et al.* (46) who found binding of an antibody directed against the platelet glycoprotein IIb/IIIa complex in 16 of 21 (75%) frozen melanoma tissues compared with no binding in 15 cryostat sections containing normal melanocytes in skin or nevi. Since glycoprotein IIb is found exclusively on platelets, it seems likely that the antibody used by these investigators was identifying

the β_3 subunit. Taken together, these observations emphasize the importance of studying the distribution of cell surface receptors, such as the integrins, on cells in their "natural," *i.e., in situ,* environment, and support the observation made here that the expression of the β_3 subunit is common in invasive melanomas.

RGP and VGP tumors behave in a well-defined and predictable manner (47). The radial growth phase is characterized by indolent but inexorable growth and by a propensity to progress to vertical growth phase. Although RGP melanomas may be invasive, they do not form nodules in the dermis, they do not metastasize to distant sites, and they are not capable of causing metastatic tumors in nude mice (36, 47). Survival, upon excision of tumors at this stage is 100%. Clinically, only those melanomas that express VGP characteristics have the capacity for metastasis (47). Since the distinction between early VGP and RGP can be difficult, the expression of the β_3 subfamily of integrins by melanoma cells with tumorigenic potential could be useful as a marker of cells entering this phase of malignant progression.

In contrast to the selective expression of the β_3 subunit by VGP and metastatic melanomas, the α_v subunit was strongly expressed on all tissues (Fig. 2*G*). This suggests that the α_v subunit associates with a different β subunit in normal melanocytes and nevi. The ability of the α_v subunit to combine with alternative β subunits is well documented (48–50). One notable difference between the α_v/β_3 receptor and the other α_v-containing receptors is that only the $\alpha_v\beta_3$ receptor has the ability to bind to fibrinogen. This property may somehow be important in the process of tumor progression.

Whether the expression of a receptor containing the β_3 subunit contributes to the capacity of melanoma cells to invade matrix and blood vessels is not known. It is possible, however, that the possession of this additional adhesion receptor allows the cells to move more freely within the mesenchymal stroma during tumor formation and invasion or bind more readily to the endothelial lining of blood vessels during the metastatic process. Evidence that this integrin subfamily may be important in tumor implantation and growth has recently been provided by Boukerche *et al.* (20) who have inhibited the growth of a human melanoma cell line in nude mice using an antibody with reactivity against the β_3 subunit of the vitronectin receptor. Further evidence that the $\alpha_v\beta_3$ receptor may be important in metastasis is provided by studies which have shown that peptides containing the RGD sequence are able to inhibit both melanoma tumor cell invasion (16) and the development of experimental metastases by murine melanomas (17–19).

In summary, this study has demonstrated that melanoma cells in culture and in tissue express a wide variety of integrins in a relatively heterogenous pattern. However, the expression of the β_3 subunit in tissue sections was restricted exclusively to tumorigenic melanoma cells. This suggests that the presence of this receptor may be important for the development of tumor invasiveness and could be useful as a marker of melanoma cells entering the more aggressive phase of the malignant process.

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